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solution)(73) Proprietor: KYOWA HAKKO KOGYO CO., LTD.
Chiyoda-ku, Tokyo 100 (JP)

(72) Inventors:

- MORI, Hideo
Tokyo 194 (JP)
- IIDA, Akihiro
Tokyo 194 (JP)
- TESHIBA, Sadao
Tokyo 194-01 (JP)
- FUJIO, Tatsuro
Yamaguchi 747 (JP)

(74) Representative: Casalonga, Axel et al
D-80469 München (DE)(56) References cited:
JP-A-57 110 194 US-A- 4 152 209

- MOLECULAR AND GENERAL GENETICS vol. 143, no. 1, 1975, Berlin, DE, pages 85 - 91, BJARNE JOCHIMSEN et al.: 'Location on the chromosome of Escherichia coli of genes governing purine metabolism'
- JOURNAL OF GENERAL MICROBIOLOGY, Vol. 135, No. 5, (1989), B. HOVE-HENSEN et al.: 'Role of Guanosine Kinase in the Utilization of Guanosine for Nucleotide Synthesis in Escherichia coli', p. 1263-1271
- CHEMICAL ABSTRACTS, Vol. 68, (1968), K.J. PIERRE, et al.: 'Formation of inosine 5'-monophosphate by a kinase in cell-free extracts of Ehrlich ascites cell invitro', Abstract No. 102069a

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The file contains technical information submitted after the application was filed and not included in this specification

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Description

Technical Field

5 The present invention relates to an inosine-guanosine kinase which catalyzes the reaction of forming 5'-inosinic acid (5'-IMP) from inosine and adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP) and the reaction of forming 5'-guanylic acid (5'-GMP) from guanosine and ATP or dATP, and to a process for producing 5'-nucleotides using the enzyme.

Background Art

10 5'-Nucleotides, inter alia, 5'-IMP and 5'-GMP have a flavor enhancing activity and are widely used as a seasoning agent. As the method for producing 5'-nucleotides, there are known a method for enzymatically decomposing RNA present in yeast cells (Journal of Agricultural and Chemical Society of Japan, 34, 489, 1969), a method for culturing a microorganism having an ability to produce 5'-IMP [Agricultural and Biological Chemistry, 46, 2557 (1982)], a method for chemical phosphorylation of nucleosides such as inosine and guanosine [Bulletin of the Chemical Society of Japan, 42, 3505-3508 (1969)], and the like. Currently, it is considered that the chemical phosphorylation utilizing these nucleosides would be the most advantageous from an industrial standpoint, because inosine and guanosine are easily obtained by fermentation. However, in the case of performing chemical phosphorylation, large quantities of chlorides are used at a lower temperature [Bulletin of the Chemical Society of Japan, 42, 3505-3508 (1969)] so that the method is not desired from viewpoints of both costs and environmental hygiene. Accordingly, enzymatic phosphorylation of nucleosides under mild conditions is desired. Hitherto, biochemical phosphorylation of purine nucleosides using cultured cells of various microorganisms has been reported (Japanese Published Unexamined Patent Application Nos. 116698/83 and 230094/88) but is inferior to chemical phosphorylation in terms of conversion rate and yield.

25 As an inosine-phosphorylating enzyme, inosine kinase (EC 2.7.1.73) is known but the crude active fraction derived from animal tissues or microorganisms is only reported [The Enzymes, Vol. IX, 54-56 (1973), Academic Press; Nucleosides and Nucleobases in Microorganisms, pp. 66, (1983), Academic Press]. The presence of a guanosine kinase activity which phosphorylates guanosine in E. coli is also suggested [J. Gen. Microbiol., 135, 1263-1273 (1989)]. However, the activity is low and it is extremely difficult to purify the enzyme so that it is far beyond industrial utilization. In addition, physicochemical properties of the enzyme are not clarified. In any event, inosine-guanosine kinase is an enzyme that has been neither isolated nor purified.

30 An object of the present invention is to provide an inosine-guanosine kinase and also to provide a method for preparing 5'-nucleotides, inter alia, 5'-IMP and 5'-GMP, which have been widely utilized as a seasoning agent, industrially at low costs.

Disclosure of the Invention

35 According to the present invention, there are provided an inosine-guanosine kinase derived from a microorganism belonging to the genus Escherichia which catalyzes the reaction of forming 5'-IMP from inosine and ATP or dATP and the reaction of forming 5'-GMP from guanosine and ATP or dATP, a process for producing the enzyme and a process for preparing 5'-nucleotides using the enzyme.

Hereafter the present invention is described in detail.

40 The inosine-guanosine kinase in accordance with the present invention can be obtained by culturing in a medium a microorganism belonging to the genus Escherichia having an ability to produce the inosine-guanosine kinase, producing and accumulating the inosine-guanosine kinase in cultured cells and recovering the inosine-guanosine kinase therefrom.

As the microorganism to be used, it may be any of the microorganisms which are obtainable from the natural world or constructed by genetic engineering so long as it belongs to the genus Escherichia and has an ability to produce the inosine-guanosine kinase.

45 Hereafter a method for obtaining the microorganism of the present invention constructed by genetic engineering and a method for producing the inosine-guanosine kinase using the microorganism are given.

The inosine-guanosine kinase may be obtained by isolating chromosomal DNA from a microorganism belonging to the genus Escherichia, cloning a gene encoding the inosine-guanosine kinase to produce a bacterial strain having an enhanced inosine-guanosine kinase activity using genetic engineering and culturing the strain.

50 Isolation and cloning of the gene encoding the inosine-guanosine kinase derived from a strain belonging to the genus Escherichia can be carried out as described below. That is, chromosomal DNA from strains of the genus Escherichia is isolated by a conventional method for isolation of DNA, for example, the phenol method [Biochim. Biophys. Acta, 72, 619-629 (1963)]. The resulting chromosomal DNA is cleaved with a suitable restriction enzyme, for example,

BamHI, Sau3AI, BglII, etc. By inserting the fragments cleaved with the restriction enzyme into a vector DNA, a recombinant DNA in which the DNA fragment containing the gene encoding the inosine-guanosine kinase derived from a strain of the genus Escherichia has been inserted can be obtained, together in a mixture with various other recombinant DNAs. In the recombinant DNA mixture described above is the recombinant DNA comprising the DNA fragment containing the gene encoding the inosine-guanosine kinase. Using the recombinant DNA mixture, a host microorganism is transformed according to the method of Cohen et al. [Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1979)].

As a source for the gene encoding the inosine-guanosine kinase, there is mentioned chromosomal DNA of a microorganism belonging to the genus Escherichia. Specific examples are Escherichia coli HM70 strain, Escherichia coli W3110 strain [Molecular Cloning, Cold Spring Harbor Laboratory (1982); ATCC 14948], etc.

As the vector harboring the DNA fragment containing the gene encoding the inosine-guanosine kinase inserted therein, any vector among phage vectors, plasmid vectors, etc. may be used as long as the vector is autonomously replicated in a strain belonging to the genus Escherichia. Preferred examples are pBR322 [Gene, 2, 95 (1977)], pUC19 [Gene, 33, 103 (1985)], pTrS30 [Doctoral Thesis by Tatsuya Nishi (1988), pp. 130, Tokyo University], etc.

Any host microorganism may be used as long as it belongs to the genus Escherichia and is capable of carrying a recombinant DNA obtained by inserting, into a vector, the gene encoding the inosine-guanosine kinase derived from a strain belonging to the genus Escherichia. Specific examples are Escherichia coli HM70 strain, Escherichia coli MC1000 strain [J. of Molecular Biology, 138, 179-207 (1980)], Escherichia coli DH1 strain [Molecular Cloning, 505, Cold Spring Harbor Laboratory (1982)], etc.

The gene may also be inserted into another bacterial species by subcloning the gene from the thus obtained recombinant DNA containing the gene encoding the inosine-guanosine kinase derived from a strain belonging to the genus Escherichia, by use of the host-vector system of the other species.

As the host-vector system, all known systems may be used. Examples include the host-vector systems of the genus Serratia, the genus Corynebacterium, the genus Brevibacterium, the genus Pseudomonas, the genus Bacillus, etc.

Escherichia coli HM70 strain which is one of the sources for the gene encoding the inosine-guanosine kinase is a strain obtained by disrupting, on a genetic level, a part of the nucleoside-degrading activity of Escherichia coli SΦ609 strain [Molec. Gen. Genet., 143, 85-91 (1975)] obtained from National Genetics Research Institute, Japan, through treatment with UV rays. The HM70 strain is used not only as a source for the gene but also as a host for cloning of the gene.

Selection of HM70 strain was made as follows. SΦ609 strain has an inosine-degrading activity and therefore, when the strain is allowed to grow on a plate medium supplemented with inosine, it degrades inosine into hypoxanthine and ribose. Furthermore, the SΦ609 strain can use ribose as a sugar source and thus, when it is allowed to grow on a sugar metabolism assay plate supplemented with inosine (MacCONKEY plate supplemented with inosine), it assimilates ribose so that red colonies are formed. Therefore, SΦ609 strain is smeared on inosine-supplemented MacCONKEY plate and UV rays are irradiated to such a degree that a killing rate becomes about 95%, whereby mutation is induced. The appearing white colonies are strains having a lowered ribose productivity, namely, strains having a decreased inosine-degrading activity. A strain having the lowest inosine-degrading activity is selected from the colonies and named HM70 strain.

SΦ609 strain and HM70 strain cannot grow on the minimum agar plate medium supplemented with hypoxanthine since these strains lack the purine nucleotide biosynthesis pathway and the salvage pathway which produces 5'-IMP from hypoxanthine. However, HM70 strain having a decreased inosine-degrading activity which is about one fourth of that of SΦ609 strain can utilize inosine. Therefore, HM70 strain can grow even on inosine-supplemented minimum agar plate medium [plate medium prepared by dissolving 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g of NaCl, 1 g of NH₄Cl and 15 g of agar in 1 liter of distilled water, adjusting pH to 7.4 with 1N NaOH, then sterilized by autoclaving and then adding a sterile solution of 2 ml of 1M MgSO₄, 10 ml of 20% glucose and 0.1 ml of 1M CaCl₂ (inosine was supplemented in a final concentration of 5 mM)], while SΦ609 strain cannot grow on that medium. However, this growth restoration results from a weak inosine-guanosine kinase activity inherently possessed by HM70 strain and its growth rate is extremely low. When inosine-guanosine kinase structural gene ligated with a plasmid vector is introduced into this HM70 strain, the inosine-guanosine kinase activity in the cells increases and growth on the inosine-supplemented minimum agar plate medium is accelerated. On the other hand, since the parent strain SΦ609 has a strong inosine-degrading activity, even though a recombinant DNA comprising the inosine-guanosine kinase gene and a plasmid vector is introduced into the parent strain, the parent strain cannot grow on inosine-supplemented minimum agar plate medium because of degradation of inosine in the medium.

From the thus obtained transformants, a strain having an activity which catalyzes the reaction of forming 5'-IMP from inosine and ATP or dATP and the reaction of forming 5'-GMP from guanosine and ATP or dATP is selected as follows.

The obtained transformants are treated with an organic solvent to afford membrane permeability thereto. Using the thus treated product as an enzyme source, the reaction of forming 5'-IMP from inosine and ATP or dATP or the reaction of forming 5'-GMP from guanosine and ATP or dATP is carried out. An amount of 5'-IMP or 5'-GMP produced is quan-

titatively determined by high performance liquid chromatography (HPLC). A strain which shows an increased amount of 5'-IMP or 5'-GMP produced is an inosine-guanosine kinase clone. Also when a transformant constructed by using HM70 strain as a host is inoculated on an inosine-supplemented minimum agar plate medium and kept at 30°C, a few colonies showing a fast growth appear in 2 to 3 days. Most of these strains showing a fast growth are inosine-guanosine kinase clones. From the obtained transformants, recombinant DNA molecules are isolated to obtain a recombinant DNA comprising the gene encoding the inosine-guanosine kinase derived from a strain belonging to the genus Escherichia.

Examples of the strains in which inosine-guanosine kinase activity has been enhanced include Escherichia coli HM70/pBM2 carrying a recombinant DNA constructed by inserting into a vector a gene encoding the inosine-guanosine kinase derived from Escherichia coli HM70 strain, Escherichia coli DHL/pBMI carrying a recombinant DNA constructed by inserting into a vector a gene encoding the inosine-guanosine kinase derived from Escherichia coli W3110, etc. Escherichia coli HM70/pBM2 and Escherichia coli DHL/pBMI have respectively been deposited as Escherichia coli HM72 and Escherichia coli HMI with the Fermentation Research Institute, Agency of Industrial Science & Technology, Japan, under the Budapest Treaty, as shown in Table 1.

Table 1

Indication of Microorganism Recognition	FERM BP-	Date of Deposit
<u>Escherichia coli</u> HM72	3125	October 6, 1990
<u>Escherichia coli</u> HMI	2669	December 1, 1989

These strains having an enhanced inosine-guanosine kinase activity are cultured in accordance with a conventional method for culturing bacteria. That is, the microorganism may be cultured in a conventional medium containing carbon sources, nitrogen sources, inorganic compounds, amino acids, vitamins, etc. under aerobic conditions; while controlling temperature, pH, etc.

As the carbon sources used in a medium, carbohydrates such as glucose, fructose, sucrose, molasses, blackstrap molasses, starch hydrolyzate, etc. alcohols such as ethanol, glycerine, sorbitol, etc., organic acids such as pyruvic acid, lactic acid, acetic acid, etc. amino acids such as glycine, alanine, glutamic acid, aspartic acid, etc. can be used so long as the microorganism can assimilate them. A concentration of these carbon sources used is preferably 5 to 30%.

As the nitrogen sources, ammonia, ammonium salts of various inorganic and organic compounds such as ammonium chloride, ammonium sulfate, ammonium nitrate, ammonium carbonate, ammonium acetate, ammonium phosphate, etc. nitrogen-containing organic compounds such as urea, peptone, NZ amine, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, fish meal or its digested products, etc. various amino acids such as glycine, glutamic acid, etc. can be used. A concentration of these nitrogen sources used is generally 0.1 to 10%.

As the inorganic compounds, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate, magnesium phosphate, sodium chloride, ferrous sulfate, manganese sulfate, zinc sulfate, calcium carbonate, etc. can be used.

When the microorganism to be used requires a specific nutrient such as amino acid, nucleic acid, vitamin, etc. for its growth, these substances are supplemented to the medium in a suitable amount.

Culturing is carried out under aerobic conditions such as shaking culture, agitation submerged culture, etc. A preferred temperature for culturing is generally 28 to 32°C. A period for culturing is generally 1 to 24 hours. A pH of medium is desirably kept neutral with ammonia, urea, a sodium hydroxide solution, etc.

After the completion of the culturing, the inosine-guanosine kinase may be isolated from the cultured cells in a conventional manner for collecting an enzyme, for example, as follows. Firstly the resulting cells are thoroughly washed and then ultrasonicated to give cell-free extract. After centrifugation, protamine sulfate is added to the resulting supernatant. The mixture is centrifuged to remove high molecular nucleic acid as precipitates. The supernatant is added to Sephadex G-50 followed by desalting through gel filtration. Subsequently, an anion exchange chromatography treatment using DEAE Sepharose and gel filtration through Sephacryl S-200 are carried out to give the purified product.

Components of each buffer in the specification refer to the following compounds

HEPES: N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
PIPES: piperazine-N,N'-bis(2-ethanesulfonic acid)
TAPS: N-tris(hydroxymethyl)methyl-3-amino-1-propanesulfonic acid
CHES: cyclohexylaminoethanesulfonic acid

The activity of the inosine-guanosine kinase obtained is determined as follows. A reaction solution (hereafter referred to as reaction solution a) having a composition of 100 mM HEPES buffer (pH 7.2), 10 mM MgSO₄, 50 mM KCl, 1 mM ATP and 1 mM inosine is brought into contact with the inosine-guanosine kinase in a concentration of about 10 µg protein/mf followed by reaction at 30°C for about 30 minutes. During the course of reaction, a part of the reaction solution

is subjected to sampling intermittently. After the reaction solution is diluted to 1/20 with 0.2M NaH_2PO_4 (adjusted the pH to 2.6 with H_3PO_4) to stop the reaction, an amount of 5'-IMP in the stopped reaction solution is quantitatively determined by HPLC. Analysis by HPLC is made by using 0.2M NaH_2PO_4 (pH 2.6) at a flow rate of 1 ml/min. as an eluting solution and Asahipak GS-320H (manufactured by Asahi Chemical Co., Ltd.) as a column. Detection of components was made by using the UV absorbance at 254 nm as an index. Quantitative determination was made by comparing absorbancy with that of the standard.

Next, physicochemical properties of the obtained inosine-guanosine kinase are described below.

(1) Action

The enzyme has an action of forming ADP and 5'-IMP from ATP and inosine, an action of forming dADP and 5'-IMP from dATP and inosine, an action of forming ADP and 5'-GMP from ATP and guanosine, and an action of forming dADP and 5'-GMP from dATP and guanosine.

(2) Optimum pH

A reaction was carried out at 30°C for 20 minutes in a manner similar to the method for determination of inosine-guanosine kinase activity described above, except that PIPES (pH 6.6-7.1), HEPES (pH 6.9-8.3), TAPS (pH 7.9-8.8) and CHES (pH 8.7-10.1) were used in replacement of the buffer component (100 mM) in the composition of reaction solution a. As the result, the optimum pH was 6.9-8.2.

(3) pH Stability

This enzyme is treated at 4°C for 16 hours in an aqueous solution containing 50 mM buffer (CHES; pH 10.0-9.0, TAPS; pH 8.2, HEPES; pH 8.3-7.3 or PIPES; pH 6.6) and 5 mM β -mercaptoethanol under both conditions in the absence of KCl and in the presence of 250 mM KCl. After the treatment, the activity is determined. When treated in a pH range of 6.6 to 9.0, the treated enzyme maintains the residual activity of 90% or more of the activity of intact standard enzyme and stably keeps its activity in a pH range of 6.6 to 9.0. The addition of KCl ameliorates storage stability.

(4) Optimum temperature

In the method for determination of inosine-guanosine kinase activity described above, the activity is determined by varying the temperature of 0 to 50°C and 31 to 41°C. The optimum temperature is 25 to 40°C.

(5) Temperature stability

A reaction solution composed of 20% glycerol, 50 mM Tris-hydrochloride buffer (pH 8), 5 mM β -mercaptoethanol and 100 mM NaCl is contacted with inosine-guanosine kinase in a concentration of about 10 μg protein/ml and treated at a temperature of 26 to 50°C for 15 minutes to determine the residual activity at each temperature. As the result, since treatment at 40°C or lower inactivates the enzyme by only 20% or less, the enzyme is stable up to 40°C. In contrast, the activity is rapidly lost by treatment at 50°C.

(6) Substrate specificity

In the method for determination of inosine-guanosine kinase activity described above, a reaction is carried out at 30°C by changing the concentration of KCl to 300 mM in the composition of reaction solution a and adding various neutralized phosphate sources to the reaction solution instead of ATP in a final concentration of 5 mM. As shown in Fig. 1, the reaction proceeds well when using ATP or dATP as substrate for phosphate-donor. Further in the case of uridine triphosphate (UTP), the reaction proceeds a little bit. However, adenosine diphosphate (ADP), adenosine monophosphate (5'-AMP), guanosine triphosphate (GTP), orotic acid monophosphate (5'-OMP), cytidine monophosphate (5'-CMP), p-nitrophenyl phosphate (PNPP), acetylphosphoric acid, tripolyphosphoric acid, tetrapolyphosphoric acid, pyrophosphoric acid and phosphoric acid could not act as phosphate-donor substrates for this enzyme in this reaction. This indicates that the enzyme is classified in an enzyme group called kinases and clearly distinguished from enzymes which govern transphosphorylation used in Japanese Published Unexamined Patent Application Nos. 119898/83 and 230094/88, etc., namely, nucleoside phosphotransferases (EC 2.7.1.77). Where ATP or dATP is used as phosphate-donor, inosine and guanosine are preferred as phosphate-acceptor.

(7) Inhibitors

In the method for determination of inosine-guanosine kinase activity described above, the activity is determined by reacting at 30°C for 30 minutes except that the concentration of KCl is changed to 300 mM and 1 mM metal salt is added to the composition of reaction solution a. The activity is determined as a standard when no metal salt is added. The results are shown in Table 2. The enzyme is inhibited by metal ions such as Co^{2+} , Cu^{2+} , Zn^{2+} , etc.

Table 2

Metal Salt (1 mM)	Relative Activity (%)
None	100
FeCl_2	112
FeCl_3	131
CaCl_2	107
CoCl_2	32
CuCl_2	1
MnCl_2	134
BaCl_2	116
ZnSO_4	5
$\text{Zn}(\text{CH}_3\text{COO})_2$	5
NaCl	104
NaF	96

(8) Activation

In the method for determination of inosine-guanosine kinase activity described above, a reaction is carried out by changing the buffer to Tris-hydrochloride buffer (pH 8.0), and varying the concentration of KCl in the composition of reaction solution a. The results are shown in Fig. 2. When 100 mM NaCl is added instead of KCl, no reaction proceeds. This reveals that the enzyme requires K^+ for activation. Next, activation is examined in the system where the concentration of KCl is changed to 300 mM in the composition of reaction solution a, by adding various divalent or trivalent metal ion salts instead of MgSO_4 . As shown in Table 3, Mg^{2+} and Mn^{2+} exhibit an activating action. It is thus revealed that this enzyme requires one of two combinations: one is K^+ and Mg^{2+} and the other is K^+ and Mn^{2+} .

Table 3

Metal Salt (10 mM)	Produced IMP (mM)
None	0
FeCl_2	0
FeCl_3	0
CaCl_2	0
CoCl_2	0.01
CuCl_2	0
MnCl_2	0.51
BaCl_2	0
ZnSO_4	0
$\text{Zn}(\text{CH}_3\text{COO})_2$	0
MgCl_2	0.60
MnSO_4	0.64
MgSO_4	0.68

(9) K_m value

K_m value in the reaction solution composed of 2 mM ATP, 10 mM MgSO_4 , 300 mM KCl and 0.1 M HEPES buffer (pH 7.2) was 2.1 mM for inosine and 6.1 μM for guanosine.

(10) Amino acid sequence and nucleotide sequence

Nucleotide sequence of the structural gene coding for this enzyme was determined by the dideoxy chain terminator method [Science, 214, 1205-1210 (1981), Gene, 19, 269-276 (1982)] and its amino acid sequence was deduced from the nucleotide sequence. The amino acid sequence and the nucleotide sequence are shown in Tables 4 and 5, respectively.

Table 4

1	MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
15	21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
	41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
	61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
20	81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
	101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
25	121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
	141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
	161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
30	181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
	201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
35	221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
	241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
	261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
40	281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
	301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
	321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
45	341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
	361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
50	381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
	401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
55	421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

Table 5

5	1	ATGAAATTTTC	CCGGTAAACG	TAAATCCAAA	CATTACTTCC	CCGTAAACGC
	51	ACGCGATCCG	CTGCTTCAGC	AATTCCAGCC	AGAAAACGAA	ACCAGCGCTG
	101	CCTGGGTAGT	GGGTATCGAT	CAAACGCTGG	TCGATATTGA	AGCGAAAGTG
10	151	GATGATGAAT	TTATTGAGCG	TTATGGATTA	AGCGCCGGGC	ATTCACTGGT
	201	GATTGAGGAT	GATGTAGCCG	AAGCGCTTTA	TCAGGAACTA	AAACAGAAAA
15	251	ACCTGATTAC	CCATCAGTTT	GCGGGTGGCA	CCATTGGTAA	CACCATGCAC
	301	AACTACTCGG	TGCTCGCGGA	CGACCGTTCC	GTGCTGCTGG	GCGTCATGTG
	351	CAGCAATATT	GAAATTGGCA	GTTATGCCTA	TCGTTACCTG	TGTAACACTT
20	401	CCAGCCGTAC	CGATCTTAAC	TATCTACAAG	GCGTGGATGG	CCCGATTGGT
	451	CGTTGCTTTA	CGCTGATTGG	CGAGTCCGGG	GAACGTACCT	TTGCTATCAG
25	501	TCCAGGCCAC	ATGAACCAGC	TGCGGGCTGA	AAGCATTCCG	GAAGATGTGA
	551	TTGCCGGAGC	CTCGGCACTG	GTTCTCACCT	CATATCTGGT	GCGTTGCAAG
	601	CCGGGTGAAC	CCATGCCGGA	AGCAACCATG	AAAGCCATTG	AGTACGCGAA
30	651	GAAATATAAC	GTACCGGTGG	TGCTGACGCT	GGGCACCAAG	TTTGTCATTG
	701	CCGAGAATCC	GCAGTGGTGG	CAGCAATTCC	TCAAAGATCA	CGTCTCTATC
35	751	CTTGCGATGA	ACGAAGATGA	AGCCGAAGCG	TTGACCGGAG	AAAGCGATCC
	801	GTTGTTGGCA	TCTGACAAGG	CGCTGGACTG	GGTAGATCTG	GTGCTGTGCA
	851	CCGCCGGGCC	AATCGGCTTG	TATATGGCGG	GCTTTACCGA	AGACGAAGCG
40	901	AAACGTAAAA	CCCAGCATCC	GCTGCTGCCG	GCGCTATAG	CGGAATTCAA
	951	CCAGTATGAG	TTTAGCCGCG	CCATGCGCCA	CAAGGATTGC	CAGAATCCGC
45	1001	TGCGTGTATA	TTCCACATT	GC3CCGTACA	TGGGCGGGCC	GGAAAAAATC
	1051	AT3AACACTA	ATGGAGCGGG	GGATGGCGCA	TTGGCAGCGT	TGCTGCATGA
	1101	CATTACCGCC	AACAGCTACC	ATCGTAGCAA	CGTACCAAAC	TCCAGCAAAC
50	1151	ATAAATTCAC	CTGGTTAACT	TATTCATCGT	TAGCGCAGGT	GTGTAAATAT
	1201	GCTAACCGTG	TGAGCTATCA	GGTACTGAAC	CAGCATTAC	CTCGTTTAAAC
55	1251	GCGCGGCTTG	CCGGAGCGTG	AAGACAGCCT	GGAAGAGTCT	TACTGGGATC
	1301	GT				

10 amino acids sequence of the enzyme at the N-terminal were determined using Amino Acid sequencer of Applied

Biosystem Co., Ltd. The amino acid sequence coincided with the amino acid sequence (Table 4) deduced from the nucleotide sequence shown in Table 5. Methionine which is the first amino acid of this enzyme is not cut out. Furthermore, C-terminal peptide was isolated and collected from peptide groups obtained by digesting the enzyme with lysyl endopeptidase and amino acid sequence of the C-terminal peptide was determined by the method described above. The amino acid sequence coincided with the C-terminal amino acid sequence shown in Table 4. The foregoing reveals that the amino acid sequence of this enzyme coincides with the amino acid sequence (Table 4) deduced from the nucleotide sequence shown in Table 5.

(11) Molecular weight

Molecular weight deduced from the amino acid sequence is about 48400 daltons; according to measurement by SDS-polyacrylamide electrophoresis (manufactured by Biorad Co., Ltd., standard for low molecular weight, catalogue No. 161-0304 was used), molecular weight is about 43000 daltons.

The nucleotide sequence shown in Table 5 corresponds to the inosine-guanosine kinase represented by the amino acid sequence shown in Table 4. However, unless the inosine-guanosine kinase activity is lost, a strain carrying a recombinant DNA inserted with a gene obtained by modifying the nucleotide sequence may similarly be used as a strain having an increased inosine-guanosine kinase activity. Even a mutated enzyme produced by modifying guanine at the 1282nd position and its subsequent nucleotide sequence as shown in Table 6 exhibits an inosine-guanosine kinase activity.

Table 6

C-Terminal of natural type

```

      1281                      1302
      ↓                        ↓
GAAGACAGCCTGGAAGAGTCTTACTGGGATCGT
GluAspSerLeuGluGluSerTyrTrpAspArg
424      427 428                      434

```

C-Terminal of mutant type

```

      1311
      ↓
GGCATGCAAGCGGGTACCGAGCTCGAATTC
GlyMetGlnAlaGlyThrGluLeuGluPhe
428                      437

      1312                      1335
      ↓                        ↓
ACTGGCCGTCGTTTTACAACGTCG
ThrGlyArgArgPheThrThrSer
438                      445

```

Next, mention may be made of process for producing 5'-nucleotides using the enzyme which catalyzes the reaction of forming 5'-nucleotides from nucleosides and ATP or dATP.

5'-nucleotides can be obtained by contacting nucleosides with ATP or dATP in an aqueous medium in the presence of an enzyme source having the enzyme activity which catalyzes the reaction of forming 5'-nucleotides from nucleosides and ATP or dATP.

As the nucleoside, inosine or guanosine is mentioned. As the 5'-nucleotides, 5'-IMP or 5'-GMP is mentioned.

As the enzyme source, inosine-guanosine kinase, bacterial cells having an ability to produce the enzyme, bacterial cells carrying a recombinant DNA obtained by inserting into a vector a DNA fragment containing a gene encoding the enzyme involved in the reaction of forming 5'-nucleotides from nucleosides and ATP or dATP, a culture thereof or treated matters thereof, etc. may be used.

As the treated matters, a concentrate of the culture or a dried culture, a culture treated with surfactant and/or organic solvent or lytic enzyme, bacterial cells obtained by centrifuging the culture, dried bacterial cells, acetone-treated cells, cells treated with surfactants and/or organic solvents, lytic enzyme-treated cells, immobilized bacterial cells or enzyme preparation extracted from bacterial cells, etc. may be used.

As the inosine and guanosine to be used in the reaction, any of purified or crude products, fermentation broth or cell-free supernatant of inosine and guanosine can be used for the reaction so long as they do not interfere the reaction

of forming 5'-nucleotides. The concentration of nucleosides is in the range of from 10 to 80 g/l.

As the phosphate donor, there are ATP or dATP. Any of purified and crude ATP and dATP or materials containing AT or dATP may be used as ATP or dATP source so long as they do not contain any substance that interferes the reaction.

Since ATP and dATP are expensive, it is advantageous to add a microorganism having an ATP regenerating activity (Japanese Published Unexamined Patent Application No. 74595/86) to the reaction system, and to synthesize ATP from glucose and inorganic phosphoric acid.

In this case, an ATP precursor, an ATP regeneration energy donor, a phosphate donor and a microorganism having an ATP biosynthesis activity are allowed to exist in the reaction solution, instead of ATP. When the reaction system coupled with the ATP regeneration system is used, it is sufficient to use ATP in a catalytic amount (1.0 g/l or less). When a necessary amount is supplied and brought into the reaction system from bacteria or a culture, it is unnecessary to particularly supplement ATP. Examples of the strains having an ATP regenerating activity are Brevibacterium ammoniagenes KY13761 strain [Agric. Biol. Chem., 42, 399-405 (1978)], Brevibacterium ammoniagenes ATCC 21477 strain, etc.

Brevibacterium ammoniagenes KY13761 produce inosine by fermentation. Therefore, when inosine fermentation broth (containing cells) obtained by culturing this KY13761 strain is used as a source of nucleoside and as a source of ATP regeneration, inosine in the fermentation broth can directly be phosphorylated without requiring purification of inosine, so that an efficient process for producing 5'-IMP can be provided in a less expensive way.

The reaction for forming 5'-nucleotides from inosine or guanosine, and ATP or dATP is carried out at a temperature of 20 to 40°C for 1 to 48 hours while adjusting pH to 6-8, preferably by adding a surfactant and/or an organic solvent. As the surfactant useful for treating the bacterial cells and for the reaction, there may be used surfactants including cationic surfactants such as polyoxyethylene stearylamine (e.g., Nymeen S-215, manufactured by Nippon Oil and Fats, Co., Ltd.; hereafter the same shall be used unless otherwise indicated), cetyltrimethylammonium bromide, cation FB, cation F2-40E, etc.; anionic surfactants such as sodium oleylamide sulfate, Newrex TAB, Rapizole 80, etc.; amphoteric surfactants such as polyoxyethylene sorbitan monostearate (e.g., Nonion ST221), etc.; tertiary amine PB, hexadecyl-dimethylamine, etc.; any other surfactants may be used so long as they accelerate the reaction of forming 5'-nucleotides from nucleosides and ATP or dATP. These surfactants may be used generally in a concentration of 0.1 to 50 mg/ml, preferably 1 to 20 mg/ml.

As the organic solvent, toluene, xylene, aliphatic alcohols, benzene, ethyl acetate may be used. The organic solvent may be used generally in a concentration of 0.1 to 50 µl/ml, preferably 1 to 20 µl/ml.

After completion of the reaction, 5'-nucleotides produced and accumulated in the reaction solution are obtained in a conventional manner using ion exchange resin, etc.

Brief Description of the Drawings

Fig. 1 relates to substrate specificity for inosine-guanosine kinase, wherein the ordinate represents an amount of 5'-TMP produced and the abscissa represents time.

Fig. 2 relates to KCl dependency of inosine-guanosine kinase, wherein the ordinate represents an amount of 5'-IMP produced and the abscissa represents an amount of KCl added.

Fig. 3 is a restriction enzyme map of plasmid pBM2 containing a gene encoding inosine-guanosine kinase derived from Escherichia coli HM70 strain.

Fig. 4 is a restriction enzyme map of mutant plasmid pBM Δ14 in which deletion in the structural gene of inosine-guanosine kinase has occurred up to the 5' end.

Fig. 5 is a restriction enzyme map of mutant plasmid pBMM5 in which deletion in the structural gene of inosine-guanosine kinase has occurred up to the 3' end.

Fig. 6 is a restriction enzyme map of vector pTrS30.

Fig. 7 is a restriction enzyme map of plasmid pIK75 which efficiently expresses inosine-guanosine kinase.

Fig. 8 shows a nucleotide sequence at the junction between the tryptophan promoter and the structural gene of inosine-guanosine kinase.

Fig. 9 is a restriction enzyme map of plasmid pBMI containing a gene encoding inosine-guanosine kinase derived from Escherichia coli W3110 strain.

Best Mode for Practising the Invention

In the following examples, reagents and vectors used in genetic engineering experiments are all manufactured by Takara Shuzo Co., Ltd. Other reagents are those manufactured by Nakarai Tesque Co., Ltd.

Example 1

(1) Isolation of DNA

Escherichia coli HM70 strain was inoculated into LB liquid medium [1% trypton, 0.5% yeast extract and 1% sodium chloride (pH 7.5)] followed by culturing at 37°C overnight. After 15 g of the cultured cells was suspended in 120 ml of 20 mM Tris-hydrochloride buffer (pH 7.5) containing 2 mM EDTA, 15 ml of lysozyme solution (20 mg/ml lysozyme was dissolved in 20 mM Tris-hydrochloride buffer (pH 7.5) containing 2 mM EDTA) was added to the suspension. The mixture was allowed to stand at 30°C for an hour. Then 15 ml of 20% sodium laurylsulfate was added thereto and the mixture was slowly stirred. Next, 150 ml of phenol saturated with 10 mM Tris-hydrochloride buffer containing 1 mM EDTA was added to the solution followed by thoroughly stirring. The solution was centrifuged and 150 ml of the aqueous layer was fractionated. Such procedure for the phenol extraction was repeated 3 times. To 150 ml of the obtained aqueous layer were added 15 ml of 2.5 M sodium acetate solution and further 300 ml of ethanol. The precipitated chromosomal DNA was wound up around a glass rod and dried. Then, chromosomal DNA was dissolved in 30 ml of 10 mM Tris-hydrochloride buffer containing 1 mM EDTA and 50 µg/ml ribonuclease was added to the solution. The mixture was allowed to stand at 37°C for 30 minutes. After the same procedure for phenol extraction as described above was performed, 3 ml of 2.5 M sodium acetate solution and 60 ml of ethanol were added to the aqueous layer followed by standing at -20°C for 16 hours. After centrifugation, the obtained pellet was washed with 70% ethanol solution and dried to give purified chromosomal DNA. The chromosomal DNA was suspended in 10 mM Tris-hydrochloride buffer containing 1 mM EDTA.

(2) Preparation of recombinant DNA

Sau3AI was added to the suspension containing 1 µg of chromosomal DNA obtained in (1) to perform partial digestion. Separately, BamHI was added to 20 µl of a solution containing 1 µg of vector pUC19 for digestion. Then, 2 µl of 1 M Tris-hydrochloride buffer (pH 8.0) was added thereto and the mixture was treated with alkaline phosphatase at 65°C for an hour. The digested chromosomal DNA and vector DNA described above were purified by the same procedures for phenol extraction and ethanol precipitation as in (1). After 100 ng of purified chromosomal DNA and 20 ng of purified vector DNA were suspended in a solution containing 66 mM Tris-hydrochloride buffer (pH 7.6), 66 mM magnesium chloride, 10 mM DTT and 0.1 mM ATP, 10 units of T4 ligase were added to the suspension. The mixture was allowed to stand at 14°C for 16 hours for the purpose of ligating chromosomal DNA with vector DNA to obtain a recombinant DNA.

(3) Preparation of an Escherichia coli strain with the recombinant DNA introduced therein

Escherichia coli HM70 strain was inoculated in 50 ml of LB liquid medium followed by culturing at 37°C for 4 hours. The bacteria harvested by centrifugation at 3000 rpm for 7 minutes were suspended at 0°C in 20 ml of 50 mM calcium chloride solution. After the suspension was allowed to stand at 0°C for 20 minutes, the cells were collected by the same centrifugation as described above and suspended at 0°C in 40 ml of 50 mM calcium chloride solution. The suspension was mixed with the solution containing the recombinant DNA obtained in (2) and the mixture was allowed to stand at 0°C for 10 minutes. After heat-treating at 42°C for 90 seconds, the mixture was smeared on minimum agar plate medium containing 50 µg/ml ampicillin and 5 mM inosine. This plate medium was kept at 30°C for 48 to 72 hours.

(4) Isolation of DNA encoding inosine-guanosine kinase

Several colonies appeared on the plate medium described in (3) in 2 to 3 days. Each of the colonies was cultured at 30°C overnight in LB liquid medium. A part of each culture was smeared on a minimum agar plate medium containing 50 µg/ml ampicillin and 5 mM inosine, which was kept at 30°C for 36 to 48 hours. On the second day a colony which grew well, namely, a transformant carrying a recombinant DNA containing the inosine-guanosine kinase gene was selected. The transformant carrying a recombinant DNA containing the inosine-guanosine kinase gene was cultured in LB liquid medium at 30°C overnight. After the cells were harvested, plasmid DNA was isolated by the method described in Maniatis et al. [Molecular Cloning (1982), Cold Spring Harbor Laboratory] and its nucleotide sequence was determined by the dideoxy method [Messing, J., Methods in Enzymology, 101, 20-78 (1983)]. The nucleotide sequence of the structural gene moiety of inosine-guanosine kinase is as shown in Table 5.

The thus obtained recombinant DNA containing the gene encoding inosine-guanosine kinase derived from Escherichia coli HM70 strain was named pBM2. A restriction enzyme map of pBM2 is shown in Fig. 3.

In Fig. 3, part indicated with black bold line is the structural gene encoding inosine-guanosine kinase derived from Escherichia coli HM70 strain. The direction of transcription of the gene is from ClaI site to BglII site in the figure.

(5) Construction of the plasmid which highly expresses inosine-guanosine kinase and its introduction into a microorganism

The structural gene of inosine-guanosine kinase is ligated downstream of tryptophan promoter of *Escherichia coli*, whereby inosine-guanosine kinase can be expressed in high efficiency.

According to Molecular Cloning (pp. 86-96), plasmid pBM2 obtained in (4) was purified. Using restriction enzymes *Sma*I (5 units) and *Kpn*I (5 units), 10 µg of pBM2 was fully digested. The resulting digested product was treated with *Exo*III nuclease to prepare a mutant in which nucleotides located in the direction of the inserted fragment are deleted. Mutant plasmid pBMΔ14 (Fig. 4) in which the deletion had occurred up to 5' end of the inosine-guanosine kinase structural gene was selected. For construction of the deletion mutant, Deletion Kit for Kilo sequencing (manufactured by Takara, Japan) was used according to the brochure attached. This plasmid pBMΔ14 was digested with *Pst*I and *Xba*I and a mutant in which nucleotides located to the direction of the inserted fragment were digested by *Exo*III nuclease, was prepared in the same way. Thus, mutant plasmid pBMM5 (Fig. 5) in which the deletion had occurred up to 3' end of the inosine-guanosine kinase structural gene was obtained. After 10 µg of pBMM5 was digested with 10 units of restriction enzyme *Hind*III, the digested end was made to be a blunt end using the DNA blunting kit. Ethanol was added to collect the DNA having a blunt end as a precipitate and this DNA was digested with restriction enzyme *Sac*I. The digested products was isolated by agarose gel electrophoresis and a fragment containing the inosine-guanosine kinase structural gene was recovered from the gel (using DNA Prep manufactured by Asahi Glass Co., Ltd.) The recovered fragment was inserted into *Sac*I-*Nru*I site of vector pTrs30 (Fig. 6) to give plasmid pIKI. Then 10 µg of pIKI was digested with 5 units of *Cla*I (*Cla*I site in the inosine-guanosine kinase structural gene does not undergo digestion since it is generally modified by methylation). After the digested product was recovered as a precipitate by addition of ethanol, deletion was introduced at both of the digestion ends using nuclease BAL31. The deletion plasmid was recovered by precipitation by addition of ethanol followed by ligation treatment (ligation kit). The deletion plasmid was used for transformation of MC1000 strain. From the obtained transformants, various deletion plasmids were obtained. From these plasmids, plasmid pIK75 (Fig. 7) in which the inosine-guanosine kinase structural gene had been ligated right downstream of the Shine-Dalgarno sequence derived from the vector was selected. The nucleotide sequence at the junction between the tryptophan promoter and the inosine-guanosine kinase structural gene is shown in Fig. 8. Genetic engineering techniques such as digestion with restriction enzymes, etc. used in this section were performed according to Molecular Cloning, unless otherwise indicated.

(6) Culture of a highly inosine-guanosine kinase-expressed microorganism

When MC1000 strain carrying plasmid pIK75 obtained in (5) [hereafter referred to as MC1000(pIK75)] is cultured, high density culture method [Biotechnol. Bioeng., 17, 227-239 (1975)] is appropriate. MC1000(pIK75) strain was cultured by the high density culture method.

MC1000(pIK75) strain was inoculated into 500 ml of seed medium having the following composition followed by culturing at 30°C for 16 hours.

Composition of seed medium: 3.5 g/l KH_2PO_4 , 3.5 g/l $(\text{NH}_4)_2\text{HPO}_4$, 1.0 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g/l glucose, 5.0 g/l yeast extract, 3 ml/l trace element solution (sterilized at 120°C for 30 minutes by steam)

Composition of trace element solution: 27 g/l $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2 g/l $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$, 2 g/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2 g/l $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g/l CuCl_2 , 0.5 g/l H_3BO_3 , 100 ml/l conc. HCl

Next, 3 l of a fermentation medium (medium obtained by further adding 10 g of $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ and 5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to 3 l of seed medium having the composition described above) was charged in a fermentation tank of 7.5 l volume followed by sterilization at 120°C for 30 minutes with steam. After 150 ml of 50% (w/v) sterile glucose solution was added to the fermentation tank, 500 ml of the seed medium in which MC1000 (pIK75) had been cultured was added.

While adjusting the pH to 6.8 with 5.5 M aqueous ammonia, culture was continued for 24 hours with agitation (6000 rpm) and aeration (3 l/min). In 4 to 6 hours after initiation of culture, a glucose concentration in the culture was reduced to 2.5% or less. From this point, 50% (w/v) glucose solution was continuously fed in the fermentation tank little by little to maintain the glucose concentration in the culture at 2 to 3%. (The culture was centrifuged and the cells recovered as precipitates were stored at -20°C. The cells were used in the following (7) and (9). Furthermore the medium after completion of the culture was frozen at -20°C and thawed at 30°C immediately before use in the following (10)).

(7) Purification of inosine-guanosine kinase

After 6 g of the -20°C-stored cells which had been obtained in (6) was suspended in 24 ml of purification buffer [20% glycerol, 50 mM Tris-hydrochloride buffer (pH 8) and 5 mM β-mercaptoethanol], the suspension was disrupted with a homogenizer (manufactured by Brown Biotech Co., Ltd., glass bead diameter of 0.1 mm). The homogenate was

centrifuged to give about 20 ml of the supernatant. Protamine sulfate was added to the supernatant in a final concentration of 0.4% followed by centrifugation. Thus high molecular nucleic acid components were removed as precipitates. The resulting supernatant was passed through Sephadex G-50 column which had been previously equilibrated with the purification buffer. The column was eluted with the purification buffer to give about 30 ml of desalted active fraction. After the fraction was passed through DEAE Sepharose column which had been previously equilibrated with the purification buffer, 60 ml of the purification buffer containing 0.1 M NaCl was added thereto. Then the desired enzyme was eluted with 200 ml of the purification buffer having an NaCl linear concentration gradient of 0.1 M to 0.6 M. From the active fractions eluted, 3 ml of the fraction having the highest enzyme concentration was collected. This active fraction was passed through Sephacryl S-200 column and eluted with the purification buffer containing 0.1 M NaCl. By gel filtration, the active fraction was collected. Finally 10 ml of solution purified enzyme (1.4 µg protein/ml) was obtained. This purified enzyme was analyzed by SDS-polyacrylamide electrophoresis and no impurity was detected. The enzyme is stably stored at -20°C in the purification buffer containing 0.1 M NaCl.

(B) Culture of a microorganism capable of regenerating ATP

Brevibacterium ammoniagenes KY13761 strain was cultured at 30°C for 2 days on a seed agar medium plate prepared by adding 25 g/l agar to a seed medium having the composition described in Table 7. The obtained cells were inoculated into 30 ml of seed medium contained in an Erlenmeyer's flask of 250 ml volume followed by shaking culture at 30°C for 24 hours.

The 30 ml of the resulting culture was inoculated into 3 l of seed medium having the composition described in Table 7, in a fermentation tank of 5 l volume. While adjusting pH to 6.8 with 5.5 M aqueous ammonia, culture was continued for 24 hours with agitation at 600 rpm and aeration of 3 l/min.

300 ml of the obtained culture was inoculated into 3 l of fermentation medium having a composition described in Table 7, in a fermentation tank of 5 l volume. While adjusting pH to 6.8 with 5.5 M aqueous ammonia, culture was continued at 32°C for 42 hours with agitation at 600 rpm and aeration of 3 l/min. [The medium was centrifuged and the cells recovered as precipitates were stored at -20°C. The cells were used in the following (9). Furthermore the medium (containing about 30 g/l of inosine) after completion of the culture was directly frozen at -20°C and thawed at 30°C immediately before use in the following (10)].

Table 7

Composition (g/l)	Seed Medium	Fermentation Medium
Glucose	50	150
KH ₂ PO ₄	1	10
K ₂ HPO ₄	3	10
MgSO ₄ · 7H ₂ O	1	10
CaCl ₂ · 2H ₂ O	0.1	0.1
FeSO ₄ · 7H ₂ O	0.01	0.01
ZnSO ₄ · 7H ₂ O	0.001	0.001
MnSO ₄ · 4-6H ₂ O	0.004	0.004
L-Cystein · HCl	0.02	0.02

Thiamine	0.005	0.005
Ca-D-pantothenate	0.01	0.01
5 Nicotinic acid	none	0.005
Biotin	30 µg/l	30 µg/l
Urea	5	2
10 (NH ₄) ₂ SO ₄	5	none
Meat extract	none	10
Polypeptone	10	none
Yeast extract	10	none
15 Adenine	0.3	0.2
pH	7.2	8.3

[used after steam sterilization (120°C, 30 minutes)]

(9) Production of 5'-nucleotides from nucleosides by resting cell reaction

While maintaining at 32°C by strongly stirring 20 ml of a solution having composition shown in Table 8, reaction was carried out for 24 hours, while keeping pH at 7.2 using 4 N NaOH. A small amount of the reaction mixture was subjected to sampling intermittently to determine concentrations of phosphoric acid, inosine and 5'-IMP in the mixture. For quantitative assay for phosphoric acid, Phospho B-test WAKO (manufactured by Wako Pure Chemical Industry Co., Ltd.) was used and a difference between measurement data every time and initial addition was complemented to keep the concentration of phosphate ion constant by adding monopotassium phosphate. Concentrations of inosine and 5'-IMP were quantitatively determined by HPLC according to a modification of the method described hereinbefore. About 100 g/l of 5'-IMP (calculated as disodium 7.5 hydrate) was produced from about 50 g/l of initial inosine 24 hours after starting the reaction. In this case, the molar conversion rate was 90% or more. When guanosine was added instead of inosine, 5'-GMP was produced in a molar conversion rate of 90% or more with similar time course.

Table 8

Composition of reaction solution	
KY13761 strain	200 g wet cell weight/l
MC1000 (pIK75) strain	20 g wet cell weight/l
Inosine	50 g/l
40 Monopotassium phosphate	20 g/l
Glucose	30 g/l
Magnesium sulfate	5 g/l
Xylene	10 ml/l
45 Nymphen S-215	4 g/l
Phytic acid	5 g/l

(10) Production of 5'-IMP from inosine using inosine fermentation medium

After completion of fermentation, about 30 g/l of inosine was accumulated in the medium obtained in (8) above. As indicated in Table 9, 20 ml of a reaction mixture containing this medium was prepared. In a manner similar to (9), while keeping at 32°C with vigorously stirring and also keeping pH at 7.2 with 4 N NaOH, a reaction was carried out for 13 hours. During the course of reaction, monopotassium phosphate was added as in (9). From 23.5 g/l of initial inosine, 46 g/l of 5'-IMP was produced and accumulated. Also in this case, its molar conversion rate was 90% or more, as in (9).

Table 9

Composition of reaction solution

Inosine culture broth	
(containing KY13761 cells)	15.7 ml
MC1000(pIK75) culture broth	1.82 ml
Monopotassium phosphate	0.4 g
Glucose	0.7 g
Magnesium sulfate	0.1 g
Xylene	0.2 ml
Nymeen S-215	80 mg
phytic acid	100 mg
(Distilled water is added to the foregoing components until 20 ml).	

Example 2

(1) Isolation of DNA

Purified chromosomal DNA was obtained in a manner similar to Example 1 (1) except for using Escherichia coli W3110 strain in place of Escherichia coli HM70 strain. The chromosomal DNA was suspended in 10 mM Tris-hydrochloride buffer containing 1 mM EDTA.

(2) Preparation of recombinant DNA

A recombinant DNA was obtained in a manner similar to Example 1 (2) except for using the suspension containing 1 µg of chromosomal DNA obtained in Example 2 (1) in place of the suspension obtained in Example 1 (1).

(3) Preparation of an Escherichia coli strain with the recombinant DNA introduced therein

Escherichia coli DHI strain was inoculated into 50 ml of LB liquid medium followed by culturing at 37°C for 4 hours. The bacteria harvested by centrifugation at 3000 rpm for 7 minutes were suspended at 0°C in 20 ml of 50 mM calcium chloride solution. The suspension was allowed to stand at 0°C for 20 minutes, the cells were collected by the same centrifugation as described above and suspended at 0°C in 40 ml of 50 mM calcium chloride solution. The suspension was mixed with the solution containing recombinant DNA obtained in Example 2 (2) and the mixture was allowed to stand at 0°C for 10 minutes. After heat-treating at 42°C for 90 seconds, the mixture was smeared on LB agar medium plate (LB liquid medium containing 1.5% agar) supplemented with 50 µg/ml of ampicillin, 0.1 mM isopropylthiogalactoside (IPTG) and 0.004% of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

The resulting white colony was cultured at 30°C overnight in 10 ml of LB liquid medium and the cells were isolated by centrifugation. These isolated cells were stored at -20°C and their inosine-guanosine kinase activity was determined by the method shown below.

(4) Isolation of DNA encoding inosine-guanosine kinase

Escherichia coli cells containing the recombinant DNA collected in Example 2 (3) were suspended (cell concentration was 100 g wet cell weight/ml) in 100 mM Tris-hydrochloride buffer (pH 8.0) containing 10 mM ATP, 10 mM inosine and 5 mM magnesium sulfate. Xylene was added to the suspension in a concentration of 10 ml/l. After thoroughly stirring, the mixture was allowed to stand at 30°C for an hour. The reaction solution was analyzed by HPLC and the amount of 5'-IMP in the reaction solution was quantitatively determined. Almost all transformants did not produce 5'-IMP but 5'-IMP-producing bacteria were obtained in a proportion of 1 out of 50,000 samples. The thus obtained 5'-IMP-pro-

ducing bacteria are transformants carrying a recombinant DNA containing an inosine-guanosine kinase gene.

The obtained transformants were cultured at 30°C overnight in LB liquid medium and the cells were collected. Thereafter plasmid DNA was isolated by the method described in Molecular cloning and its nucleotide sequence was determined by the dideoxy method. The nucleotide sequence of the structural gene moiety of inosine-guanosine kinase is as shown in Table 5.

The thus obtained recombinant DNA containing the gene encoding inosine-guanosine kinase derived from *Escherichia coli* W3110 strain was named pBMI. A restriction enzyme map of pBMI is shown in Fig. 9.

(5) Preparation of 5'-IMP

Transformant *Escherichia coli* HMI (FERM BP-2669) transformed with recombinant DNA pBMI obtained in Example 2 (4) was cultured at 30°C for 16 hours in 400 ml of LB liquid medium (containing 50 µg/ml of ampicillin). The medium was then centrifuged to recover the cells. For control, the culture cells of *Escherichia coli* DH1/pUC19 containing vector pUC19 alone were obtained in a manner similar to the above. After 20 ml of a solution of Table 10 containing the obtained cells was reacted at 30°C for an hour, the amount of 5'-IMP produced in the solution was quantitatively determined by HPLC and found to be 5 mg. When the cells from the strain carrying the vector for control were used, no 5'-IMP was detected in the solution.

Table 10

Composition of reaction solution	
Cell	100.g wet cell weight/l
Inosine	10 mM
ATP	10 mM
Tris-hydrochloride buffer (pH 8.0)	100 mM
Magnesium sulfate	5 mM
Xylene	10 ml/l

(6) Preparation of 5'-GMP

Transformant *Escherichia coli* HMI carrying recombinant DNA pBMI obtained in Example 2 (4) was cultured at 30°C for 16 hours in 400 ml of LB liquid medium (containing 50 µg/ml ampicillin). The medium was then centrifuged to recover the cells. For control, the culture cells of *Escherichia coli* DH1/pUC19 containing vector pUC19 alone were obtained in a manner similar to the above. After 20 ml of a solution of Table 11 containing the obtained cells was incubated at 30°C for an hour, the amount of 5'-GMP produced in the solution was quantitatively determined by HPLC and found to be 2 mg. When the cell of the strain carrying the vector for control was used, no 5'-GMP was detected in the solution.

Table 11

Composition of reaction solution	
Cell	100 g wet cell weight/l
Guanosine	10 mM
ATP	10 mM
Tris-hydrochloride buffer (pH 8.0)	100 mM
Magnesium sulfate	5 mM
Xylene	10 ml/l

(7) Amplification of activity by genetic engineering

By ligating SD sequence and strong promoter sequence upstream the inosine-guanosine kinase structural gene by genetic engineering technique, expression of inosine-guanosine kinase can be amplified. The amplification can be made as follows.

After 20 µl of a solution containing 1 µg of plasmid pBMI obtained in Example 2 (4) was fully digested with 10 units of *Bam*HI, 10 units of *Sac*I and 10 units of *Sal*I, 2.8 kb fragment containing the inosine-guanosine kinase structural gene was isolated and purified by agarose gel electrophoresis (Molecular Cloning). After the purified fragment was digested with BAL31 nuclease at 37°C for 10 minutes, phenol extraction and ethanol precipitation were performed to obtain a DNA fragment having deletion at the end. Separately, 10 units of *Sma*I was added to 20 µl of a solution

containing 1 µg of vector pUC19 for full digestion. Then 2 µl of 1 M Tris-hydrochloride buffer (pH 8.0) was added and further 5 units of alkaline phosphatase was added thereto followed by reaction at 65°C for an hour. The purified inosine-guanosine kinase gene-containing DNA fragment (100 ng) and 20 ng of alkaline phosphatase-treated vector DNA were suspended in a solution containing 66 mM Tris-hydrochloride buffer (pH 7.6), 66 mM magnesium chloride, 10 mM DTT and 0.1 mM ATP. To the suspension were added 10 units of T4 ligase. The mixture was reacted at 14°C for 16 hours to ligate both DNAs, thereby to obtain a recombinant DNA. The recombinant DNA was introduced into Escherichia coli DH1 strain in a manner similar to Example 2 (3). The obtained transformants were cultured in a manner similar to Example 2 (3). The inosine-guanosine kinase activity was determined in a manner similar to Example 2 (4) and a strain having a high 5'-IMP productivity was selected. The strain was named Escherichia coli 3M100 strain. In recombinant plasmid carried by Escherichia coli BM100 strain, lactose promoter sequence was ligated upstream the inosine-guanosine kinase structural gene in a correct direction.

(8) Preparation of 5'-IMP

Escherichia coli BM100 strain was cultured in 400 ml of LB liquid medium (containing 50 µg/ml ampicillin) at 30°C for 16 hours. Centrifugation gave the cells. After 20 ml of a solution given by Table 12 containing the obtained cells and a strain having ATP regenerating activity (Japanese Published Unexamined Patent Application No. 74595/86) was incubated at 30°C for 20 hours at pH of 7.6, the amount of 5'-IMP produced in the solution was quantitatively assayed and found to be 25 g/l (calculated as the hydrate). Also in the case of using a strain carrying a recombinant DNA containing a DNA molecule, in which part of the structural gene, i.e., the DNA sequence coding for the amino-acid sequence subsequent to the 8th Leu from the C terminal is modified to code for the following amino acid sequence:

GlyMetGlnAlaGlyThrGluLeuGluPheThrGlyArgArgPheThrThrSer,

a similar titer can be obtained.

Table 12

Composition of reaction solution

Cells of <u>Brevibacterium ammoniagenes</u>	200 g wet cell
ATCC21477 (ATP-regenerating cell)	weight/l
Inosine-guanosine kinase	25 g wet cell
amplified <u>Escherichia coli</u>	weight/l
Inosine	12.5 g/l
Monopotassium phosphate	20 g/l
Glucose	50 g/l
Magnesium sulfate	5 g/l
Xylene	10 ml/l

Industrial Applicability

According to the present invention, inosine-guanosine kinase derived from a microorganism belonging to the genus Escherichia can be provided. In addition, clarification of various properties of the enzyme for the first time has led to industrial utilization thereof. Furthermore, DNA encoding the enzyme has been isolated. Using as a catalyst a microorganism containing the recombinant DNA obtained by introducing this DNA into a vector, 5'-nucleotides, e.g., 5'-IMP or 5'-GMP, can be produced from nucleosides, e.g., inosine or guanosine, in a high conversion rate.

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. A purified inosine-guanosine kinase having the following physicochemical properties:

(1) Action

The enzyme has an action of forming adenosine diphosphate (ADP) and 5'-inosinic acid (5'-IMP) from adenosine triphosphate (ATP) and inosine, an action of forming deoxyadenosine diphosphate (dADP) and 5'-IMP from deoxyadenosine triphosphate (dATP) and inosine, an action of forming ADP and 5'-guanylic acid (5'-GMP) from ATP and guanosine, and an action of forming dADP and 5'-GMP from dATP and guanosine.

(2) Optimum pH

6.9 - 8.2

(3) pH stability

The enzyme is stably kept in a pH range of 6.6 to 9.0 by treatment at 4°C for 16 hours. The addition of KCl ameliorates stability during storage.

(4) Optimum temperature

25 - 40°C

(5) Temperature stability

Stable up to 40°C by treatment at pH of 8.0 for 15 minutes

(6) Substrate specificity

Phosphoric acid at the γ -position of ATP or dATP used as phosphate donors is transferred to inosine or guanosine used as phosphate acceptors.

(7) Inhibitors

Metal ions of Co^{2+} , Cu^{2+} and Zn^{2+}

(8) Activation

The enzyme requires one of the two ion groups: one is K^+ and Mg^{2+} , and the other K^+ and Mn^{2+} .

(9) Km value

Km value in the reaction solution composed of 2 mM ATP, 10 mM MgSO_4 , 300 mM KCl and 0.1 M HEPES buffer (pH 7.2) is 2.1 mM for inosine and 6.1 μM for guanosine.

(10) Molecular weight

Molecular weight deduced from the amino acid sequence is about 48,400 daltons; according to measurement by SDS-polyacrylamide electrophoresis, molecular weight is about 43,000 daltons.

2. A purified inosine-guanosine kinase represented by the following amino acid sequence:

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 5 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 10 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 15 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 20 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 25 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 30 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 35 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 40 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

3. A gene encoding an inosine-guanosine kinase having the amino acid sequence as defined in claim 2.

4. The gene as claimed in claim 3, wherein said gene is shown by the following nucleotide sequence:

1 ATGAAATTTTC CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
5 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
10 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAACTA AAACAGAAAA
251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
301 AACTACTCGG TGCTCGCGGA CGACCGTTCTG GTGCTGCTGG GCGTCATGTG
15 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
20 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
501 TCCAGGCCAC ATGAACCAGC TCGGGGCTGA AAGCATTCCG GAAGATGTGA
25 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
601 CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACGCGAA
651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCATTG
30 701 CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
751 CTTGCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
35 801 GTTGTTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG GGCGCTATAG CGGAATTCAA
40 951 CCAGTATGAG TTTAGCCGCG CCATGCGCCA CAAGGATTGC CAGAATCCGC
1001 TGCGTGTATA TTCGCACATT GCGCCGTACA TGGGCGGGCC GGAAAAAATC
45 1051 ATGAACACTA ATGGAGCGGG GGATGGCGCA TTGGCAGCGT TGCTGCATGA
1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
50 1151 ATAAATTCAC CTGGTTAACT TATTCATCGT TAGCGCAGGT GTGTAAATAT
1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAC
1251 GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGAGTCT TACTGGGATC
55 1301 GT

5. The gene as claimed in claim 3 or 4, wherein said gene is derived from Escherichia coli.
6. A recombinant DNA obtained by inserting into a vector a DNA fragment containing a gene as defined by any one of claims 3 to 5.
7. A microorganism carrying a recombinant DNA as defined by claim 6.
8. The microorganism as claimed in claim 7, wherein said microorganism is Escherichia coli.
9. A process for preparing an inosine-guanosine kinase which comprises culturing in a medium a microorganism belonging to the genus Escherichia and carrying a recombinant DNA as defined in claim 6 to produce and accumulate the inosine-guanosine kinase in the cultured cells and collecting the inosine-guanosine kinase from the culture.
10. A process for preparing 5' -IMP or 5' -GMP which comprises reacting inosine or guanosine with a phosphate donor in an aqueous medium in the presence of an enzyme source which is (1) a purified inosine-guanosine kinase as defined in claim 1 or 2 or (2) a cell, a culture broth or a treated matter of the microorganism as defined by claim 7 to produce and accumulate 5' -IMP or 5' -GMP in the reaction mixture, and collecting 5' -IMP or 5' -GMP from the reaction mixture.
11. The process for preparing 5' -IMP or 5' -GMP as claimed in claim 10, wherein said phosphate donor is ATP or dATP.
12. The process for preparing 5' -IMP or 5' -GMP as claimed in claim 10, wherein said microorganism is a microorganism belonging to the genus Escherichia.

Claims for the following Contracting State : ES

1. A process for preparing an inosine-guanosine kinase which comprises culturing in a medium a microorganism belonging to the genus Escherichia and carrying a recombinant DNA obtained by inserting into a vector a DNA fragment containing a gene encoding an inosine-guanosine kinase having following amino acid sequence.

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

2. The process according to claim 1 characterized in that an inosine-guanosine kinase is prepared having the following physicochemical properties :

(1) Action

The enzyme has an action of forming adenosine diphosphate (ADP) and 5'-inosinic acid (5'-IMP) from adenosine triphosphate (ATP) and inosine. an action of forming deoxyadenosine diphosphate (dADP) and 5'-IMP from deoxyadenosine triphosphate (dATP) and inosine. an action of forming ADP and 5'-guanylic acid (5'-GMP) from ATP and guanosine. and an action of forming dADP and 5'-GMP from dATP and guanosine.

(2) Optimum pH

6.9 - 8.2

(3) pH stability

The enzyme is stably kept in a pH range of 6.6 to 9.0 by treatment at 4°C for 16 hours. The addition of KCl

ameliorates stability during storage.

(4) Optimum temperature

25 - 40°C

(5) Temperature stability

Stable up to 40°C by treatment at pH of 8.0 for 15 minutes

(6) Substrate specificity

Phosphoric acid at the γ -position of ATP or dATP used as a phosphate donors is transferred to inosine or guanosine used as phosphate acceptors,

(7) Inhibitor

Metal ions of Co^{2+} , Cu^{2+} and Zn^{2+}

(8) Activation

The enzyme requires one of the two ion groups: one is K^{+} and Mg^{2+} , and the other K^{+} and Mn^{2+} .

(9) Km value

Km value in the reaction solution composed of 2 mM ATP, 10 mM MgSO_4 , 300 mM KCl and 0.1 M HEPES buffer (pH 7.2) is 2.1 mM for inosine and 6.1 μM for guanosine.

(10) Molecular weight

Molecular weight deduced from the amino acid sequence is about 48,400 daltons; according to measurement by SDS-polyacrylamide electrophoresis, molecular weight is about 43,000 daltons.

3. The process according to claim 1 characterized in that the gene contained in the DNA fragment inserted into the vector has following nucleotide sequence.

1 ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 5 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
 101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 151 GATGATGAAT TTATTGACCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
 10 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAACTA AAACAGAAAA
 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 15 301 AACTACTCGG TGCTCGCGGA CGACCGTTCC GTGCTGCTGG GCGTCATGTG
 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
 20 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
 501 TCCAGGCCAC ATGAACCAGC TCCGGGCTGA AAGCATTCCG GAAGATGTGA
 25 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
 601 CCGGGTGAAC CCATGCCCGA AGCAACCATG AAAGCCATTG AGTACGGCAA
 651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCATTG
 30 701 CCGAGAATCC CCAGTGGTGG CAGCAATTCC TCAAGATCA CGTCTCTATC
 751 CTTGCGATGA ACCAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
 35 801 GTTGTTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
 851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG GCGGCTATAG CCGAATTCAA
 40 951 CCAGTATGAG TTTAGCCCGG CCATGCGCCA CAAGGATTGC CAGAATCCGC
 1001 TCGGTGTATA TTCCACATT GCGCCGTACA TCGGCGGGCC GGAATAATC
 45 1051 ATGAACACTA ATGGAGCGGG CGATGCCGCA TTGGCAGCGT TGCTCCATCA
 1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 50 1151 ATAAATTAC CTGTTAACT TATTCATCGT TAGCGCAGGT GTGTAAATAT
 1201 GCTAACCCTG TGACCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAC
 1251 CCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGACTCT TACTGGGATC
 55 1301 GT

4. A process for preparing a gene encoding an inosine-guanosine kinase comprising the steps of cloning and isolating

a gene encoding an inosine guanosine kinase having the amino acid sequence as defined in claim 1.

5. The process according to claim 4 characterized in that the gene cloned and isolated is shown by the following nucleotide sequence :

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1 ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACG/A ACCAGCGCTG
 5 101 CCGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
 10 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGA ACTA AACAGAAAA
 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 301 AACTACTCGG TGCTCGCGGA CGACCGTTCC GTGCTGCTGG GCGTCATGTG
 15 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
 20 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
 501 TCCAGGCCAC ATGAACCAGC TCGGGGCTGA AAGCATTCCG GAAGATGTGA
 25 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
 601 CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACGCGAA
 651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCATTG
 30 701 CCGAGAATCC GCAGTGGTGG CACCAATTCC TCAAAGATCA CGTCTCTATC
 751 CTTGCCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
 35 801 GTTGTTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
 851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG GGCGCTATAG CGGAATTCAA
 40 951 CCAGTATGAG TTTAGCCGCG CCATGCCGCA CAAGGATTCC CAGAATCCGC
 1001 TCGGTGTATA TTGCGACATT GCGCCGTACA TGGGCGGGCC GGA AAAATC
 45 1051 ATGAACACTA ATGAGCCGGG GGATGCCGCA TTGGCAGCGT TGCTGCATGA
 1101 CATTACCGCC AACAGGTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 50 1151 ATAAATTCAC CTGCTTA ACT TATTCATCGT TACCGCAGGT GTGTAAATAT
 1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAC
 1251 GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGAGTCT TACTGGGATC
 55 1301 GT

6. The process claimed in claim 4 or 5, wherein said gene is derived from Eschericia coli.

7. A process for producing a recombinant DNA comprising the steps of inserting into a vector a DNA fragment containing a gene as prepared according to anyone of claims 4 to 6.
8. A process for preparing 5' -IMP or 5' -GMP which comprises reacting inosine or guanosine with a phosphate donor in an aqueous medium in the presence of an enzyme source which is (1) an inosine-guanosine kinase as defined in claim 1 or (2) a cell, a culture broth or a treated matter of the microorganism carrying a recombinant DNA as defined in claim 7 to produce and accumulate 5' -IMP or 5' -GMP in the reaction mixture, and collecting 5' -IMP or 5' -GMP from the reaction mixture.
9. The process for preparing 5' -IMP or 5' -GMP as claimed in claim 8, wherein said phosphate donor is ATP or dATP.
10. The process for preparing 5' -IMP or 5' -GMP as claimed in claim 8, wherein said microorganism is a microorganism belonging to the genus Escherichia.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. Gereinigte Inosin-Guanosin-Kinase mit den folgenden physikalisch-chemischen Eigenschaften:

(1) Wirkung

Das Enzym katalysiert die Bildung von Adenosindiphosphat (ADP) und 5'-Inosinsäure (5'-IMP) aus Adenosintriphosphat (ATP) und Inosin, die Bildung von Desoxyadenosindiphosphat (dADP) und 5'-IMP aus Desoxyadenosintriphosphat (dATP) und Inosin, die Bildung von ADP und 5'-Guanylsäure (5'-GMP) aus ATP und Guanosin und die Bildung von dADP und 5'-GMP aus dATP und Guanosin,

(2) pH-Optimum

6,9 - 8,2,

(3) pH-Stabilität

das Enzym ist stabil in einem pH-Bereich von 6,6 bis 9,0 bei einer 16stündigen Behandlung bei 4°C, die Zugabe von KCl verbessert die Stabilität während der Lagerung,

(4) Temperaturoptimum

25-40°C,

(5) Temperaturstabilität

stabil bis zu 40°C bei einer 15minütigen Behandlung bei pH 8,0,

(6) Substratspezifität

Phosphorsäure an der γ -Position von ATP oder dATP als Phosphatdonor wird auf Inosin oder Guanosin als Phosphatakzeptoren übertragen,

(7) Inhibitoren

Metallionen von Co^{2+} , Cu^{2+} und Zn^{2+} ,

(8) Aktivierung

das Enzym erfordert eine der beiden Ionengruppen: die eine ist K^{+} und Mg^{2+} und die andere ist K^{+} und Mn^{2+} .

(9) Km-Wert

Der Km-Wert in der Reaktionslösung, die zusammengesetzt ist aus 2 mM ATP, 10 mM MgSO_4 , 300 mM KCl und 0.1 M HEPES-Puffer (pH 7,2) beträgt 2.1 mM für Inosin und 6.1 μM für Guanosin.

(10) Molekulargewicht

das Molekulargewicht, das von der Aminosäuresequenz abgeleitet ist, beträgt etwa 48 400 Daltons, eine Messung durch SDS-Polyacrylamidelektrophorese ergibt ein Molekulargewicht von etwa 43 000 Daltons.

2. Gereinigte Inosin-Guanosin-Kinase mit der folgenden Aminosäuresequenz:

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 5 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 10 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 15 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 20 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 25 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 30 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 35 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 40 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

- 45 3. Gen. das eine Inosin-Guanosin-Kinase mit der Aminosäuresequenz gemäß der Definition in Anspruch 2 codiert.
 4. Gen nach Anspruch 3, wobei das Gen die folgende Nucleotidsequenz aufweist:

1 ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
 5 101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
 10 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAACTA AAACAGAAAA
 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 301 AACTACTCGG TGCTCCCGGA CGACCGTTCC GTGCTGCTCG GCGTCATGTG
 15 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCGGATTGGT
 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
 20 501 TCCAGGCCAC ATGAACCAGC TGCGGGCTGA AAGCATTCCG GAAGATGTGA
 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
 601 CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACCGGAA
 25 651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCAATTG
 701 CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
 30 751 CTTGCCATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
 801 GTTGTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
 851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 35 901 AAACGTAAAA CCCAGCATCC GCTGCTGCGG GCGGCTATAG CGGAATTCAA
 951 CCAGTATGAG TTTAGCCGCG CCATGCGCCA CAAGGATTGC CAGAATCCCG
 1001 TGGGTGTATA TTCGCACATT GCGCCGTACA TGGGCGGGCC GGAAAAATC
 40 1051 ATGAACACTA ATGGAGCGGG GGATGGCGCA TTGGCAGCGT TGCTGCATGA
 1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 45 1151 ATAAATTAC CTGGTTAACT TATTCATCGT TAGCCGAGGT GTGTAAATAT
 1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAAC
 1251 GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGACTCT TACTCGGATC
 50 1301 GT

5. Gen nach Anspruch 3 oder 4, wobei das Gen von *Escherichia coli* stammt.

55 6. Rekombinante DNA, erhalten durch Insertion eines ein Gen gemäß der Definition in einem der Ansprüche 3 bis 5 enthaltenden DNA-Fragments in einen Vektor.

7. Mikroorganismus, der eine rekombinante DNA gemäß der Definition in Anspruch 6 trägt.

8. Mikroorganismus nach Anspruch 7, wobei der Mikroorganismus *Escherichia coli* ist.
9. Verfahren zur Herstellung einer Inosin-Guanosin-Kinase, umfassend die Züchtung eines Mikroorganismus, der zur Gattung *Escherichia* gehört und eine rekombinante DNA gemäß der Definition in Anspruch 6 trägt, in einem Medium zur Erzeugung und Anreicherung der Inosin-Guanosin-Kinase in den gezüchteten Zellen und die Gewinnung der Inosin-Guanosin-Kinase aus der Kultur.
10. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP, umfassend die Umsetzung von Inosin oder Guanosin mit einem Phosphatdonor in einem wäßrigen Medium in Gegenwart einer Enzymquelle, die (1) eine gereinigte Inosin-Guanosin-Kinase gemäß der Definition in Anspruch 1 oder 2 oder (2) eine Zelle, eine Kulturbrühe oder ein nach Behandlung der Mikroorganismen gemäß der Definition in Anspruch 7 erhaltenes Produkt ist, zur Erzeugung und Anreicherung von 5'-IMP oder 5'-GMP im Reaktionsgemisch und Gewinnung von 5'-IMP oder 5'-GMP aus dem Reaktionsgemisch.
11. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP nach Anspruch 10, wobei der Phosphatdonor ATP oder dATP ist.
12. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP nach Anspruch 10, wobei der Mikroorganismus ein Mikroorganismus der Gattung *Escherichia* ist.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung einer Inosin-Guanosin-Kinase, umfassend die Züchtung eines Mikroorganismus, der zur Gattung *Escherichia* gehört und eine rekombinante DNA trägt, die durch Insertion eines ein Gen, das eine Inosin-Guanosin-Kinase mit der folgenden Aminosäuresequenz codiert, enthaltenden DNA-Fragments in einen Vektor erhalten wurde, in einem Medium:

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß eine Inosin-Guanosin-Kinase mit den folgenden physikalisch-chemischen Eigenschaften hergestellt wird:

(1) Wirkung

Das Enzym katalysiert die Bildung von Adenosindiphosphat (ADP) und 5'-Inosinsäure (5'-IMP) aus Adenosintriphosphat (ATP) und Inosin, die Bildung von Desoxyadenosindiphosphat (dADP) und 5'-IMP aus Desoxyadenosintriphosphat (dATP) und Inosin, die Bildung von ADP und 5'-Guanylsäure (5'-GMP) aus ATP und Guanosin und die Bildung von dADP und 5'-GMP aus dATP und Guanosin.

(2) pH-Optimum

6.9 - 8.2.

(3) pH-Stabilität

das Enzym ist stabil in einem pH-Bereich von 6.6 bis 9.0 bei einer 16stündigen Behandlung bei 4°C, die Zugabe von KCl verbessert die Stabilität während der Lagerung.

(4) Temperaturoptimum

25-40°C,

(5) Temperaturstabilität

stabil bis zu 40°C bei einer 15minütigen Behandlung bei pH 8,0,

(6) Substratspezifität

Phosphorsäure an der γ -Position von ATP oder dATP als Phosphatdonor wird auf Inosin oder Guanosin als Phosphatakzeptoren übertragen,

(7) Inhibitoren

Metallionen von Co^{2+} , Cu^{2+} und Zn^{2+} ,

(8) Aktivierung

das Enzym erfordert eine der beiden Ionengruppen: die eine ist K^+ und Mg^{2+} und die andere ist K^+ und Mn^{2+} ,

(9) K_m -Wert

Der K_m -Wert in der Reaktionslösung, die zusammengesetzt ist aus 2 mM ATP, 10 mM MgSO_4 , 300 mM KCl und 0,1 M HEPES-Puffer (pH 7,2) beträgt 2,1 mM für Inosin und 6,1 μM für Guanosin,

(10) Molekulargewicht

das Molekulargewicht, das von der Aminosäuresequenz abgeleitet ist, beträgt etwa 48 400 Daltons, eine Messung durch SDS-Polyacrylamidelektrophorese ergibt ein Molekulargewicht von etwa 43 000 Daltons.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Gen, das in dem in den Vektor inserierten DNA-Fragment enthalten ist, die folgende Nucleotidsequenz aufweist:

1 ATGAAATTTT CCGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 5 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
 10 101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 15 151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
 20 201 GATTGAGGAT GATGTAGCCG AACCGCTTTA TCAGGAACTA AAACAGAAAA
 25 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 30 301 AACTACTCGG TGCTCGCCGA CGACCGTTCC GTGCTGCTGG GCGTCATGTG
 35 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 40 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG CCGTGGATGG CCCGATTGGT
 45 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
 50 501 TCCAGGCCAC ATGAACCAGC TCGGGGCTGA AAGCATTCCG GAAGATGTGA
 55 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTCCAAG
 60 601 CCGGGTGAAC CCATGCCCGA AGCAACCATG AAAGCCATTG AGTACCGGAA
 65 651 GAAATATAAC GTACCGGTGG TGCTGACGGT GGGCACCAAG TTTGTCATTG
 70 701 CCCAGAATCC GCAGTGGTGG CAGCAATTCC TCAAGATCA CGTCTCTATC
 75 751 CTTCCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAACCGATCC
 80 801 GTTGTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTCTGTGCA
 85 851 CCGCCGGCCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 90 901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG CCGGCTATAG CCGAATTCAA
 95 951 CCAGTATGAG TTTAGCCCGG CCATGCCCCA CAAGGATTGC CAGAATCCGC
 100 1001 TCGGTGTATA TTCCACATT GCGCCGTACA TGGCCGGGCC GGA AAAAATC
 105 1051 ATGAACACTA ATGGACGGG GGATGCCGCA TTGCCAGCGT TGCTECATGA
 110 1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 115 1151 ATAAATTAC CTGCTTAAC TATTATCGT TAGCCGAGGT GTGTAAATAT
 120 1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAAC
 125 1251 GCGCGGCTTC CCGGAGCGTG AAGACACCTT GGAAGACTCT TACTGGGATC
 130 1301 GT

4. Verfahren zur Herstellung eines eine Inosin-Guanosin-Kinase codierenden Gens, umfassend die Clonierung und Isolierung eines Gens, das eine Inosin-Guanosin-Kinase mit der Aminosäuresequenz gemäß der Definition in Anspruch 1 codiert.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß das clonierte und isolierte Gen durch die folgende Nucleotidsequenz gezeigt wird:

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5      1   ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
      51   ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
     101   CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
     151   GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
     201   GATTGAGCAT GATGTAGCCG AACCGCTTTA TCAGGAATAA AAACAGAAAA
     251   ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
     301   AACTACTCGG TGCTCGCGGA CGACCGTTTCG GTGCTGCTGG GCGTCATGTG
     351   CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
     401   CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
     451   CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
     501   TCCAGGCCAC ATGAACCAGC TCGGGGCTGA AAGCATTCCG GAAGATGTGA
     551   TTGCCGGAGC CTGGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
     601   CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACCGGAA
     651   GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTTCATTG
     701   CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
     751   CTTGCCATGA ACCAAGATGA AGCCGAAGCG TTGACCGGAG AAGCCGATCC
     801   GTTGTGGCCA TCTGACAAGG CGGTGGACTG GGTAGATCTG GTGCTGTCCA
     851   CCGCCGGGCC AATCGGCTTG TATATGGCCG GCTTTACCGA AGACGAAGCG
     901   AAACGTAAAA CCCAGCATCC GCTGCTGCCG GCGGCTATAG CCGAATTCAA
     951   CCAGTATGAG TTTAGCCCGG CCATGCGCCA CAAGGATTGC CAGAATCCCG
    1001   TCGGTGTATA TTCCGACATT GCGCCGTACA TGGCCGGCCC CGAAAAATC
    1051   ATGAACACTA ATGGAGCGGG GGATGCGCCA TTGGCAGCGT TGCTGCATGA
    1101   CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
    1151   ATAAATTAC CTGGTTAACT TATTCATCGT TAGCCGAGGT GTGTAAATAT
    1201   GCTAACCCTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTGTTTTAAC
    1251   GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGACTCT TACTGGGATC
    1301   GT

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6. Verfahren nach Anspruch 4 oder 5, wobei das Gen von *Escherichia coli* stammt.

7. Verfahren zur Herstellung einer rekombinanten DNA, umfassend die Insertion eines DNA-Fragments, das ein nach einem der Verfahren nach den Ansprüchen 4 bis 6 hergestelltes Gen enthält, in einen Vektor.
8. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP, umfassend die Umsetzung von Inosin oder Guanosin mit einem Phosphatdonor in einem wäßrigen Medium in Gegenwart einer Enzymquelle, die (1) eine Inosin-Guanosin-Kinase gemäß der Definition in Anspruch 1 oder (2) eine Zelle, eine Kulturbrühe oder ein nach Behandlung der Mikroorganismen, die eine rekombinante DNA gemäß der Definition in Anspruch 7 tragen, erhaltenes Produkt ist, zur Erzeugung und Anreicherung von 5'-IMP oder 5'-GMP im Reaktionsgemisch und Gewinnung von 5'-IMP oder 5'-GMP aus dem Reaktionsgemisch.
9. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP nach Anspruch 8, wobei der Phosphatdonor ATP oder dATP ist.
10. Verfahren zur Herstellung von 5'-IMP oder 5'-GMP nach Anspruch 8, wobei der Mikroorganismus ein Mikroorganismus der Gattung Escherichia ist.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. Inosine-guanosine kinase purifiée, présentant les propriétés physico-chimiques suivantes :

- a) action : cette enzyme exerce une action de formation d'adénosine-diphosphate (ADP) et d'acide 5'-inosinique (5'-IMP) à partir d'adénosine-triphosphate (ATP) et d'inosine, une action de formation de désoxyadénosine-diphosphate (dADP) et de 5'-IMP à partir de désoxyadénosine-triphosphate (dATP) et d'inosine, une action de formation d'ADP et d'acide 5'-guanylique (5'-GMP) à partir d'ATP et de guanosine, et une action de formation de dADP et de 5'-GMP à partir de dATP et de guanosine ;
- b) pH optimal : 6,9-8,2 ;
- c) stabilité vis-à-vis du pH : cette enzyme est stable dans le domaine de pH allant de 6,6 à 9,0, lors d'un traitement à 4°C pendant 16 heures ; l'addition de KCl améliore la stabilité en cours de conservation ;
- d) température optimale : 25-40°C ;
- e) stabilité vis-à-vis de la température : cette enzyme est stable jusqu'à 40°C, lors d'un traitement de 15 minutes à pH 8,0 ;
- f) spécificité pour le substrat : le résidu d'acide phosphorique situé en position γ dans l'ATP ou le dATP, utilisés comme donneurs de phosphate, est transféré sur l'inosine ou la guanosine, utilisées comme accepteurs de phosphate ;
- g) inhibiteurs : ions métalliques Co^{2+} , Cu^{2+} et Zn^{2+} ;
- h) activation : cette enzyme a besoin de l'une des deux paires d'ions suivantes : (K^+ , Mg^{2+}) et (K^+ , Mn^{2+}) ;
- i) valeur de K_m : dans un mélange réactionnel contenant 2 mM d'ATP, 10 mM de MgSO_4 , 300 mM de KCl et 0,1 M de tampon HEPES (pH 7,2), K_m vaut 2,1 mM dans le cas de l'inosine, et 6,1 μM dans le cas de la guanosine ;
- j) masse moléculaire : la masse moléculaire déduite de la séquence d'acides aminés vaut environ 48400 daltons, et d'après les mesures effectuées par électrophorèse en gel de polyacrylamide-SDS, la masse moléculaire vaut environ 43000 daltons.

2. Inosine-guanosine kinase purifiée, représentée par la séquence suivante de résidus d'acides aminés :

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 5 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 10 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 15 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 20 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 25 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 30 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 35 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 40 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

3. Gène codant une inosine-guanosine kinase présentant la séquence de résidus d'acides aminés indiquée dans la revendication 2.
 4. Gène conforme à la revendication 3. qui présente la séquence suivante de nucléotides :

1 ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 5 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
 10 101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 15 151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTACTGGT
 20 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAACTA AAACAGAAAA
 25 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 30 301 AACTACTCGG TGCTCGCGGA CGACCGTTCC GTGCTGCTGG GCGTCATGTG
 35 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 40 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
 45 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGGTATCAG
 50 501 TCCAGGCCAC ATGAACCAGC TCGGGCTGA AAGCATTCCG GAAGATGTGA
 55 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
 60 601 CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACGCGAA
 65 651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCAATTG
 70 701 CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
 75 751 CTGCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
 80 801 GTTGTTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
 85 851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 90 901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG GGCGCTATAG CGGAATTCAA
 95 951 CCAGTATGAG TTTAGCCGCG CCATGCGCCA CAAGGATTGC CAGAATCCGC
 100 1001 TCGGTGTATA TTCGCACATT GCGCCGTACA TGGGCGGGCC GGAAAAAATC
 105 1051 ATGAACACTA ATGGAGCGGG GGATGGCGCA TTGGCAGCGT TGCTGCATGA
 110 1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 115 1151 ATAAATTCAC CTGGTTAACT TATTCATCGT TAGCGCAGGT GTGTAAATAT
 120 1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAAC
 125 1251 GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGAGTCT TACTGGGATC
 130 1301 GT

5. Gène conforme à la revendication 3 ou 4, qui provient d'Escherichia coli.

6. ADN recombiné obtenu par insertion, dans un vecteur, d'un fragment d'ADN contenant un gène conforme à l'une des revendications 3 à 5.

7. Microorganisme hébergeant un ADN recombiné conforme à la revendication 6.
8. Microorganisme conforme à la revendication 7, appartenant à l'espèce Escherichia coli.
- 5 9. Procédé de préparation d'une inosine-guanosine kinase, lequel procédé comporte le fait de cultiver, dans un milieu, un microorganisme appartenant au genre Escherichia et hébergeant un ADN recombiné conforme à la revendication 6, de façon à produire et laisser s'accumuler de l'inosine-guanosine kinase dans les cellules cultivées, et le fait de récupérer l'inosine-guanosine kinase dans la culture.
- 10 10. Procédé de préparation de 5'-IMP ou de 5'-GMP, lequel procédé comporte le fait de faire réagir de l'inosine ou de la guanosine avec un donneur de phosphate, dans un milieu aqueux et en présence d'une source d'enzyme qui est (1) une inosine-guanosine kinase purifiée conforme à la revendication 1 ou 2, ou (2) des cellules, un bouillon de culture ou un produit de traitement d'un microorganisme conforme à la revendication 7, de façon à produire et laisser s'accumuler du 5'-IMP ou du 5'-GMP dans le mélange réactionnel, et le fait de récupérer le 5'-IMP ou le 15 5'-GMP dans le mélange réactionnel.
11. Procédé de préparation de 5'-IMP ou de 5'-GMP, conforme à la revendication 10, dans lequel ledit donneur de phosphate est de l'ATP ou du dATP.
- 20 12. Procédé de préparation de 5'-IMP ou de 5'-GMP, conforme à la revendication 10, dans lequel ledit microorganisme est un microorganisme appartenant au genre Escherichia.

Revendications pour l'Etat Contractant suivant : ES

- 25 1. Procédé de préparation d'une inosine-guanosine kinase, lequel procédé comporte le fait de cultiver, dans un milieu, un microorganisme appartenant au genre Escherichia et hébergeant un ADN recombiné obtenu par insertion, dans un vecteur, d'un fragment d'ADN qui contient un gène codant une inosine-guanosine kinase présentant la séquence suivante de résidus d'acides aminés :
30

1 MetLysPheProGlyLysArgLysSerLysHisTyrPheProValAsnAlaArgAspPro
 21 LeuLeuGlnGlnPheGlnProGluAsnGluThrSerAlaAlaTrpValValGlyIleAsp
 5 41 GlnThrLeuValAspIleGluAlaLysValAspAspGluPheIleGluArgTyrGlyLeu
 61 SerAlaGlyHisSerLeuValIleGluAspAspValAlaGluAlaLeuTyrGlnGluLeu
 81 LysGlnLysAsnLeuIleThrHisGlnPheAlaGlyGlyThrIleGlyAsnThrMetHis
 10 101 AsnTyrSerValLeuAlaAspAspArgSerValLeuLeuGlyValMetCysSerAsnIle
 121 GluIleGlySerTyrAlaTyrArgTyrLeuCysAsnThrSerSerArgThrAspLeuAsn
 15 141 TyrLeuGlnGlyValAspGlyProIleGlyArgCysPheThrLeuIleGlyGluSerGly
 161 GluArgThrPheAlaIleSerProGlyHisMetAsnGlnLeuArgAlaGluSerIlePro
 181 GluAspValIleAlaGlyAlaSerAlaLeuValLeuThrSerTyrLeuValArgCysLys
 20 201 ProGlyGluProMetProGluAlaThrMetLysAlaIleGluTyrAlaLysLysTyrAsn
 221 ValProValValLeuThrLeuGlyThrLysPheValIleAlaGluAsnProGlnTrpTrp
 25 241 GlnGlnPheLeuLysAspHisValSerIleLeuAlaMetAsnGluAspGluAlaGluAla
 261 LeuThrGlyGluSerAspProLeuLeuAlaSerAspLysAlaLeuAspTrpValAspLeu
 281 ValLeuCysThrAlaGlyProIleGlyLeuTyrMetAlaGlyPheThrGluAspGluAla
 30 301 LysArgLysThrGlnHisProLeuLeuProGlyAlaIleAlaGluPheAsnGlnTyrGlu
 321 PheSerArgAlaMetArgHisLysAspCysGlnAsnProLeuArgValTyrSerHisIle
 341 AlaProTyrMetGlyGlyProGluLysIleMetAsnThrAsnGlyAlaGlyAspGlyAla
 35 361 LeuAlaAlaLeuLeuHisAspIleThrAlaAsnSerTyrHisArgSerAsnValProAsn
 381 SerSerLysHisLysPheThrTrpLeuThrTyrSerSerLeuAlaGlnValCysLysTyr
 40 401 AlaAsnArgValSerTyrGlnValLeuAsnGlnHisSerProArgLeuThrArgGlyLeu
 421 ProGluArgGluAspSerLeuGluGluSerTyrTrpAspArg

2. Procédé conforme à la revendication 1, caractérisé en ce que l'on prépare une inosine-guanosine kinase purifiée qui présente les propriétés physico-chimiques suivantes :

a) action : cette enzyme exerce une action de formation d'adénosine-diphosphate (ADP) et d'acide 5'-inosinique (5'-IMP) à partir d'adénosine-triphosphate (ATP) et d'inosine, une action de formation de désoxyadénosine-diphosphate (dADP) et de 5'-IMP à partir de désoxyadénosine-triphosphate (dATP) et d'inosine, une action de formation d'ADP et d'acide 5'-guanylique (5'-GMP) à partir d'ATP et de guanosine, et une action de formation de dADP et de 5'-GMP à partir de dATP et de guanosine :

b) pH optimal : 6.9-8.2 :

c) stabilité vis-à-vis du pH: cette enzyme est stable dans le domaine de pH allant de 6.6 à 9.0, lors d'un traitement à 4°C pendant 16 heures l'addition de KCl améliore la stabilité en cours de conservation :

d) température optimale : 25-40°C :

e) stabilité vis-à-vis de la température: cette enzyme est stable jusqu'à 40°C, lors d'un traitement de 15 minutes à pH 8.0 :

f) spécificité pour le substrat: le résidu d'acide phosphorique situé en position γ dans l'ATP ou le dATP, utilisés

comme donneurs de phosphate, est transféré sur l'inosine ou la guanosine, utilisées comme accepteurs de phosphate ;

g) inhibiteurs: ions métalliques Co^{2+} , Cu^{2+} et Zn^{2+} ;

h) activation : cette enzyme a besoin de l'une des deux paires d'ions suivantes : (K^+ , Mg^{2+}) et (K^+ , Mn^{2+}) ;

i) valeur de K_m : dans un mélange réactionnel contenant 2 mM d'ATP, 10 mM de MgSO_4 , 300 mM de KCl et 0,1 M de tampon HEPES (pH 7,2), K_m vaut 2,1 mM dans le cas de l'inosine, et 6,1 μM dans le cas de la guanosine ;

j) masse moléculaire : la masse moléculaire déduite de la séquence d'acides aminés vaut environ 48400 daltons, et d'après les mesures effectuées par électrophorèse en gel de polyacrylamide-SDS, la masse moléculaire vaut environ 43000 daltons.

3. Procédé conforme à la revendication 1., caractérisé en ce que le gène contenu dans le fragment d'ADN inséré dans le vecteur présente la séquence suivante de nucléotides :

1 ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
 5 51 ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
 101 CCTGGGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
 151 GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
 10 201 GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAAC TA AACAGAAAA
 251 ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
 301 AACTACTCGG TGCTCGCGGA CGACCGTTCTG GTGCTGCTGG GCGTCATGTG
 15 351 CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
 401 CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
 451 CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGCTATCAG
 20 501 TCCAGGCCAC ATGAACCAGC TGCGGGCTGA AAGCATTCCG GAAGATGTGA
 551 TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
 25 601 CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACCGGAA
 651 GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCAATTG
 701 CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
 30 751 CTTGCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
 801 GTTGTGGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
 851 CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
 35 901 AAACGTAAAA CCCAGCATCC GCTGCTGCCG GCGCTATAG CGGAATTCAA
 951 CCAGTATGAG TTTAGCCGCG CCATGCCGCA CAAGGATTGC CAGAATCCGC
 40 1001 TCGGTGTATA TTGCGACATT GCGCCGTACA TGGGCGGGCC GGAAAAATC
 1051 ATGAACACTA ATGGAGCGGG GGATGGCGCA TTGGCAGCGT TGCTGCATGA
 1101 CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
 45 1151 ATAAATTCAC CTGGTTAACT TATTCATCGT TAGCGCAGGT GTGTAAATAT
 1201 GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTAC CTCGTTTAAAC
 1251 GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGAGTCT TACTGGGATC
 50 1301 GT

4. Procédé de préparation d'un gène codant une inosine-guanosine kinase, lequel procédé comporte les étapes con-
 55 sistant à cloner et à isoler un gène codant une inosine-guanosine kinase présentant la séquence de résidus d'acides
 aminés indiquée dans la revendication 1.

5. Procédé conforme à la revendication 4, caractérisé en ce que le gène cloné et isolé est représenté par la séquence

suivante de nucléotides :

```

      1  ATGAAATTTT CCGGTAAACG TAAATCCAAA CATTACTTCC CCGTAAACGC
5      51  ACGCGATCCG CTGCTTCAGC AATTCCAGCC AGAAAACGAA ACCAGCGCTG
      101  CCTGCGTAGT GGGTATCGAT CAAACGCTGG TCGATATTGA AGCGAAAGTG
10     151  GATGATGAAT TTATTGAGCG TTATGGATTA AGCGCCGGGC ATTCACTGGT
      201  GATTGAGGAT GATGTAGCCG AAGCGCTTTA TCAGGAATAA AAACAGAAAA
      251  ACCTGATTAC CCATCAGTTT GCGGGTGGCA CCATTGGTAA CACCATGCAC
15     301  AACTACTCGG TGCTCGCGGA CGACCGTTCG GTGCTGCTGG GCGTCATGTG
      351  CAGCAATATT GAAATTGGCA GTTATGCCTA TCGTTACCTG TGTAACACTT
      401  CCAGCCGTAC CGATCTTAAC TATCTACAAG GCGTGGATGG CCCGATTGGT
20     451  CGTTGCTTTA CGCTGATTGG CGAGTCCGGG GAACGTACCT TTGGTATCAG
      501  TCCAGGCCAC ATGAACCAGC TGCGGGCTGA AAGCATTCCG GAAGATGTGA
      551  TTGCCGGAGC CTCGGCACTG GTTCTCACCT CATATCTGGT GCGTTGCAAG
25     601  CCGGGTGAAC CCATGCCGGA AGCAACCATG AAAGCCATTG AGTACCGCAA
      651  GAAATATAAC GTACCGGTGG TGCTGACGCT GGGCACCAAG TTTGTCAATTG
30     701  CCGAGAATCC GCAGTGGTGG CAGCAATTCC TCAAAGATCA CGTCTCTATC
      751  CTTCCGATGA ACGAAGATGA AGCCGAAGCG TTGACCGGAG AAAGCGATCC
      801  GTTGTTGGCA TCTGACAAGG CGCTGGACTG GGTAGATCTG GTGCTGTGCA
35     851  CCGCCGGGCC AATCGGCTTG TATATGGCGG GCTTTACCGA AGACGAAGCG
      901  AAACGTAAAA CCCAGCATCC GCTGCTGCCG GCGCTATAG CGGAATTCAA
40     951  CCAGTATGAG TTTAGCCGCG CCATGCGCCA CAAGGATTGC CAGAATCCCG
1001  TCGGTGTATA TTCGCACATT GCGCCGTACA TGGGCGGGCC GGAAAAAATC
1051  ATGAACACTA ATGGAGCGGG GGATGGCGCA TTGGCAGCGT TGCTGCATGA
45     1101  CATTACCGCC AACAGCTACC ATCGTAGCAA CGTACCAAAC TCCAGCAAAC
      1151  ATAAATTACAC CTGGTTAACT TATTCATCGT TAGCGCAGGT GTGTAAATAT
      1201  GCTAACCGTG TGAGCTATCA GGTACTGAAC CAGCATTACAC CTCGTTTAAC
50     1251  GCGCGGCTTG CCGGAGCGTG AAGACAGCCT GGAAGAGTCT TACTGGGATC
      1301  GT

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- 55 6. Procédé conforme à la revendication 4 ou 5, dans lequel ledit gène dérive d'Escherichia coli.
7. Procédé de production d'un ADN recombiné, qui comporte les étapes consistant à insérer, dans un vecteur, un fragment d'ADN contenant un gène préparé conformément à l'une des revendications 4 à 6.

8. Procédé de préparation de 5'-IMP ou de 5'-GMP, lequel procédé comporte le fait de faire réagir de l'inosine ou de la guanosine avec un donneur de phosphate, dans un milieu aqueux et en présence d'une source d'enzyme qui est (1) une inosine-guanosine kinase définie dans la revendication 1, ou (2) des cellules, un bouillon de culture ou un produit de traitement d'un microorganisme hébergeant un ADN recombiné préparé conformément à la revendication 7, de façon à produire et laisser s'accumuler du 5'-IMP ou du 5'-GMP dans le mélange réactionnel, et le fait de récupérer le 5'-IMP ou le 5'-GMP dans le mélange réactionnel.
9. Procédé de préparation de 5'-IMP ou de 5'-GMP, conforme à la revendication 8, dans lequel ledit donneur de phosphate est de l'ATP ou du dATP.
10. Procédé de préparation de 5'-IMP ou de 5'-GMP, conforme à la revendication 8, dans lequel ledit microorganisme est un microorganisme appartenant au genre Escherichia.

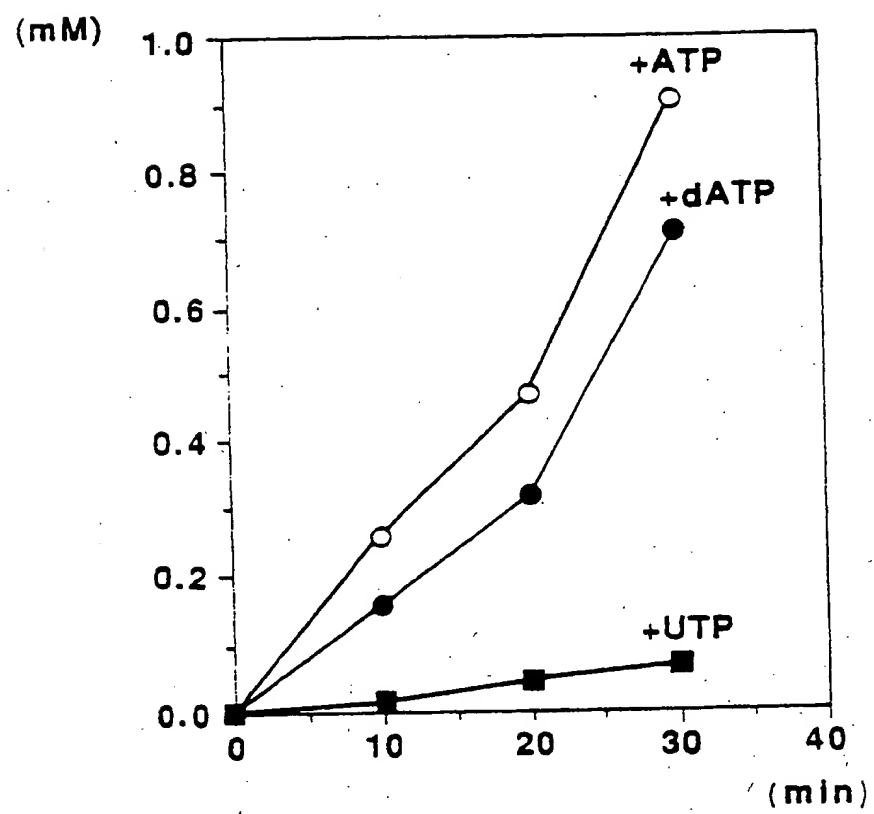
FIG.1

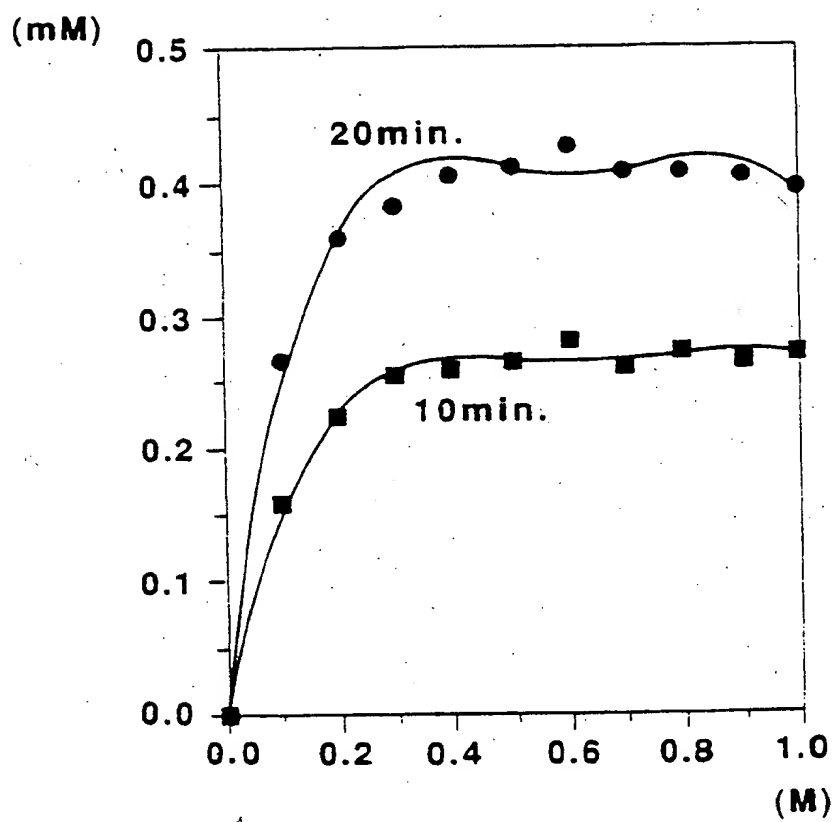
FIG. 2

FIG. 3

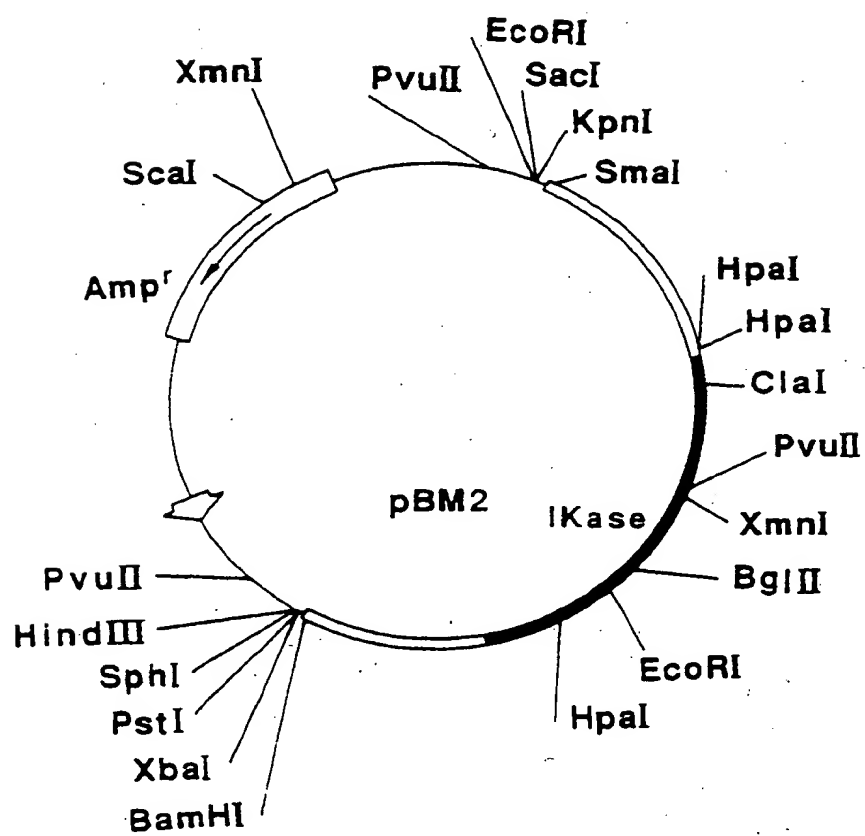


FIG. 4

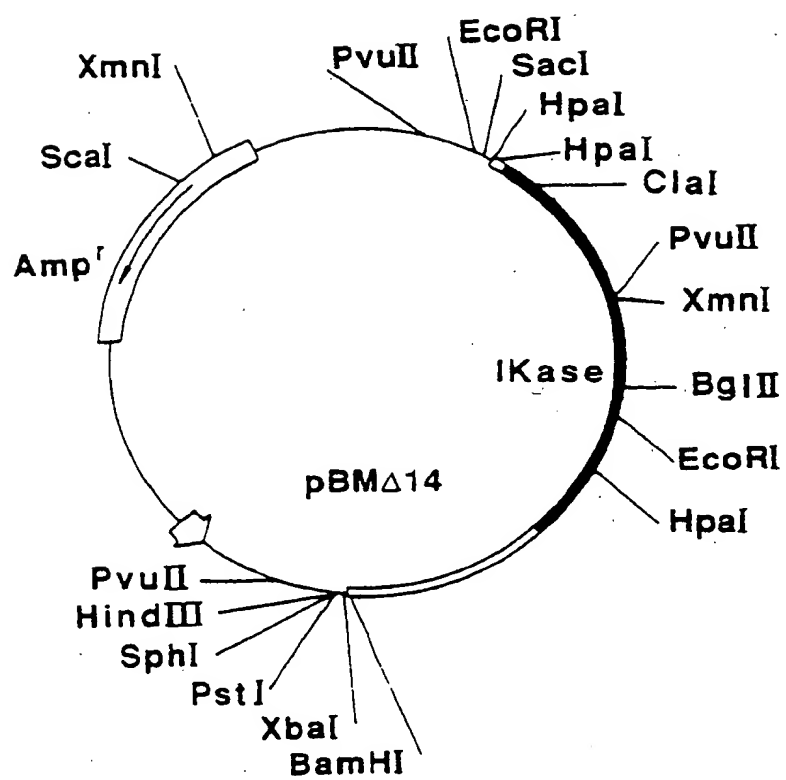


FIG. 5

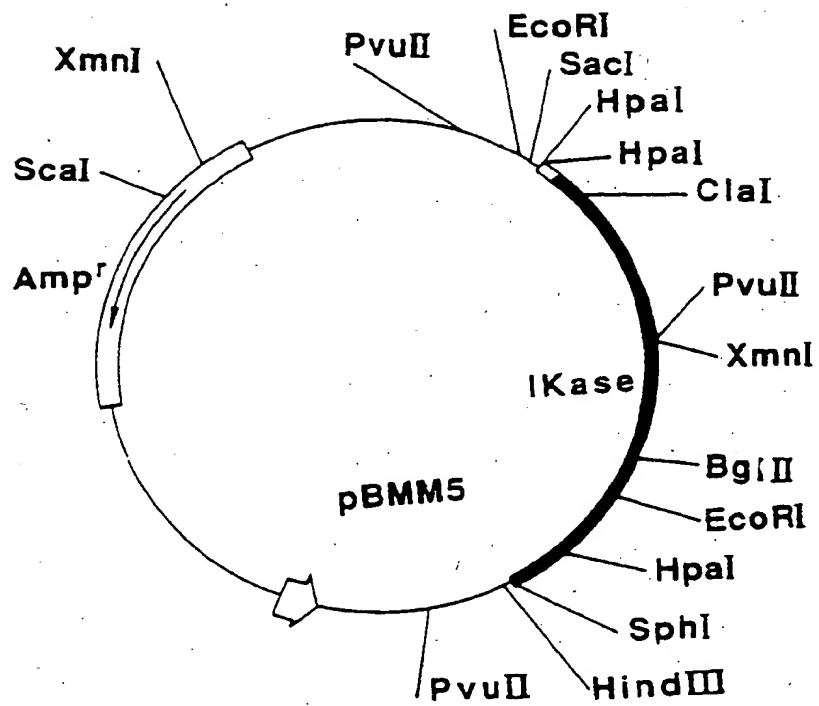


FIG. 6

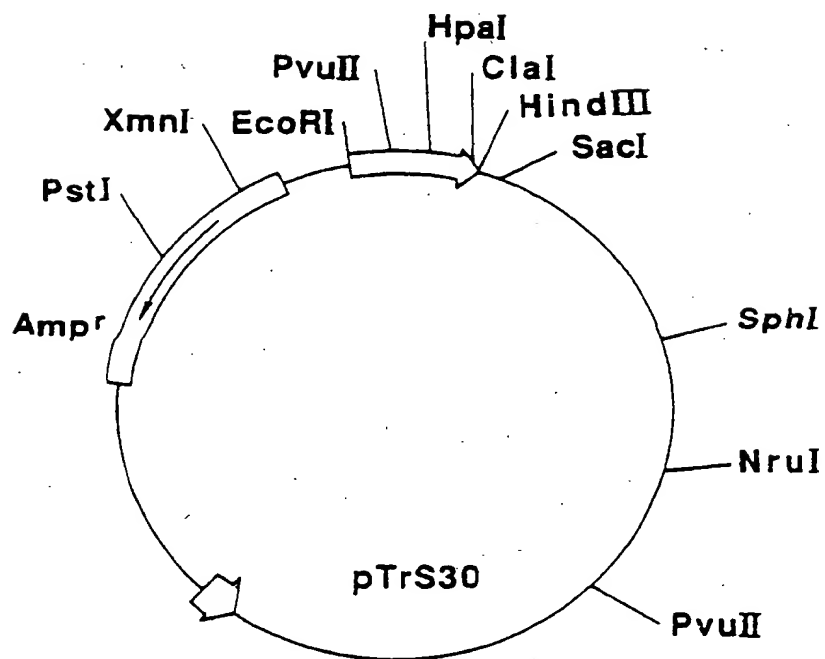


FIG. 7

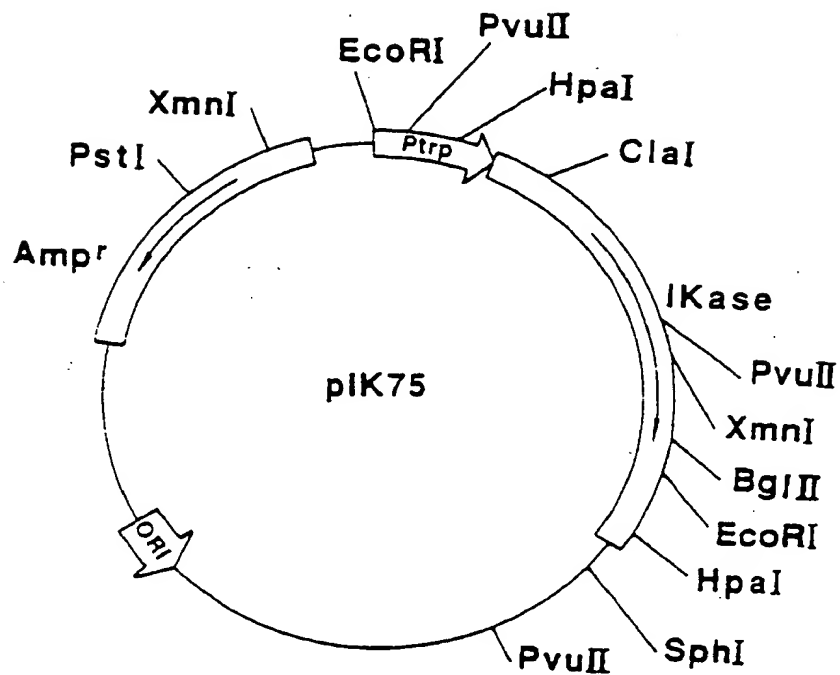


FIG. 8

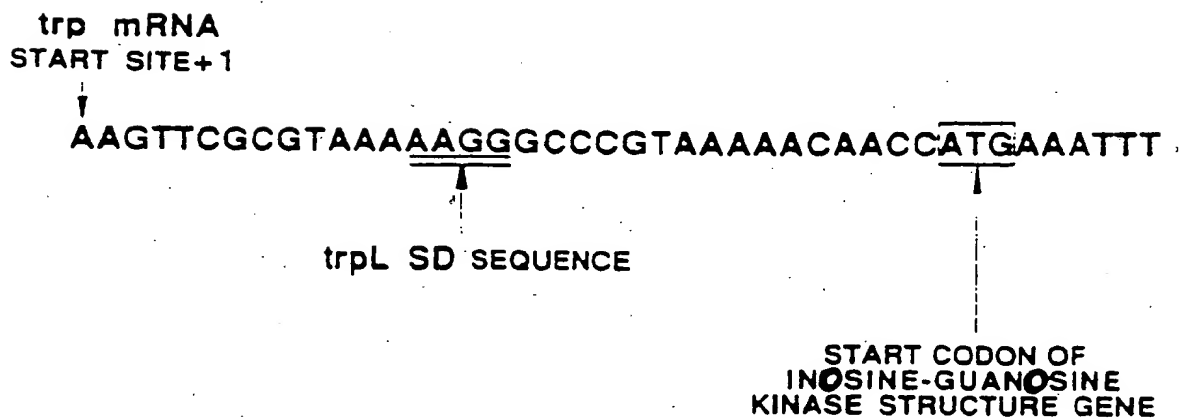


FIG. 9

